FACTORS AFFECTING FOLLICLE AND OOCYTE DEVELOPMENT IN CATTLE

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ABSTRACT

The mechanisms governing development of mammalian oocytes are not well understood. Isolation and in vitro growth of immature cattle follicles will enable determination of the factors affecting bovine follicular development, have potential applications in assisted reproduction and provide a suitable model for studying human infertility. Intercommunication of the oocyte and somatic cells is necessary for normal oocyte and follicle development. Studies using systems where oocyte-somatic cell communication is preserved allows an accurate assessment of the factors affecting follicular development. The aims of this project were to examine early follicle and oocyte development in cattle and determine whether the bovine oocyte plays a role in follicular development.

A non-enzymatic isolation procedure was developed which allowed intact bovine follicles to be isolated. On the basis of follicle size, these could be divided into 3 distinct stages: large preantral, large preantral/early antral and antral follicles. A culture technique was devised which supported in vitro follicle and oocyte development, the key elements of which were: volume of medium (0.25 ml/follicle), serum and insulin, minimal number of medium changes and a substrate of collagen.

The effect of FSH on preantral to early antral follicles in culture was examined. Initial experiments on large preantral/early antral follicle growth found that all FSH doses stimulated an increase in follicle diameter. The dose of FSH was important as low levels did not stimulate proliferation or effect oocyte size whilst high levels reduced proliferation, inhibited oocyte growth and reduced oocyte quality. Oocyte localised granulosa cell proliferation was observed in some follicles only when a healthy oocyte was present, demonstrating the importance of oocyte-somatic cell communication in granulosa cell proliferation and differentiation. The intensity of oocyte localised proliferation was reduced at high FSH doses,

confirming its dose dependent inhibitory effect on follicular development. FSH stimulated the growth of large preantral/early antral and antral follicles but not oocyte growth in any of the stages. The increase in size was due to an increase in intercellular spacing and, as antral cavities were neither maintained or formed during culture, this may be analogous to antrum development. FSH maintained granulosa cell proliferation in all follicle size classes. No detectable effect of FSH on preantral follicles were found, therefore the effect of FSH depends on the stage of follicle examined.

Apoptosis is thought to be how large follicles become atretic. It is not known if apoptosis underlies atresia in preantral follicles. Isolation of distinct follicle classes allowed apoptosis in preantral to early antral follicles to be examined in detail. Follicles isolated and selected for culture did not show apoptotic DNA fragmentation, confirming their suitability as a starting material for in vitro studies. Culture of follicles in conditions which induce apoptosis in isolated granulosa cells, did not result in apoptosis. It is possible that the oocyte has a modulating influence on follicular apoptosis or that cell death may occur by an alternative mechanism.

To further examine the role of the oocyte in directing somatic cell function, its effect on cumulus cell expansion was examined. Using intact bovine cumulus oocyte complexes or oocytectomised complexes (OOX), it was found that the oocyte is not essential for bovine cumulus cell expansion. Murine OOX complexes, which require an oocyte secreted factor for cumulus expansion, expanded in the presence of bovine oocytes Thus some factor secreted exclusively by the oocyte enabled expansion of murine OOX complexes. The purpose of the factor in bovine follicle development is unknown.

In conclusion, the isolation and culture system developed has a practical application for evaluating the effect of factors on follicular development. FSH was examined here, the effect of which depended on both dosage and stage of follicle

studied. Possible roles of bovine oocytes in granulosa cell proliferation, differentiation and apoptosis were identified. Species specific differences in the regulation of somatic cell function by the oocyte were demonstrated, underlining the need for more detailed studies in larger mammals.

PUBLICATION ARISING FROM WORK IN THIS THESIS

Published papers

Ralph, JH, Telfer, EE, Wilmut, I. Bovine cumulus cell expansion does not depend on the presence of an oocyte secreted factor. Molecular Reproduction and Development 1995; 42:248-253

Abstracts of oral presentations

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John H Ralph, Ian Wilmut, Evelyn E Telfer. Bovine ovarian follicle growth in vitro: The effect of FSH on preantral follicles of different sizes. Proceedings of the 1st UK National Ovarian Workshop, Royal Free Hospital School of Medicine, London, 24-26th November, 1995; Abstract 1.

Abstracts of poster presentations

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ABBREVIATIONS

bFGF	Basic fibroblast growth factor
cAMP	Cyclic adenosine mono phosphate
COC	Cumulus cell-oocyte complex
DMSO	Dimethyl sulphoxide
EGF	Epidermal growth factor
FCS	Foetal calf serum
FSH	Follicle stimulating hormone
GDF-9	Growth differentiation factor 9
GOC	Granulosa cell-oocyte complex
IGF	Insulin like growth factor
LH	Luteinising hormone
oFSH	ovine FSH
OOX	Oocytectomised complex
TGF	Transforming growth factor

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To Mum and Dad.

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CHAPTER 1: INTRODUCTION

1.1. OVERVIEW OF FOLLICULAR DEVELOPMENT

The production of a mature oocyte is the result of a lengthy developmental process. The different stages of follicular development can be characterised morphologically.

1.1.1. Primordial follicles

During foetal development, the cessation of primordial germ cell proliferation, entry into meiosis and subsequent arrest limits the size of the female germ cell pool. Association of primordial germ cells with flattened pre-granulosa cells marks the formation of the primordial follicle, the largest follicle pool in the ovary (Erickson, 1966) (figure 1.1.). Apart from the maintenance of the oocyte and the surrounding cells, these follicles can be regarded as dormant and may remain in this state for the entire reproductive life span of the female (review: McLaren, 1988). Enormous depletion of the primordial follicle pool occurs around birth, puberty and during illness, in addition to the continuous recruitment into the growing population (Erickson, 1966, reviewed by Gougeon, 1996). Despite many years of research, the fundamental question of what actually initiates follicle growth remains to be answered (review: McLaren, 1988). Recent studies have shown the initiation of murine and bovine primordial follicle growth in vitro (Eppig and O'Brien, 1996, Wandji et al., 1996b) although the nature of the signal has yet to be defined.

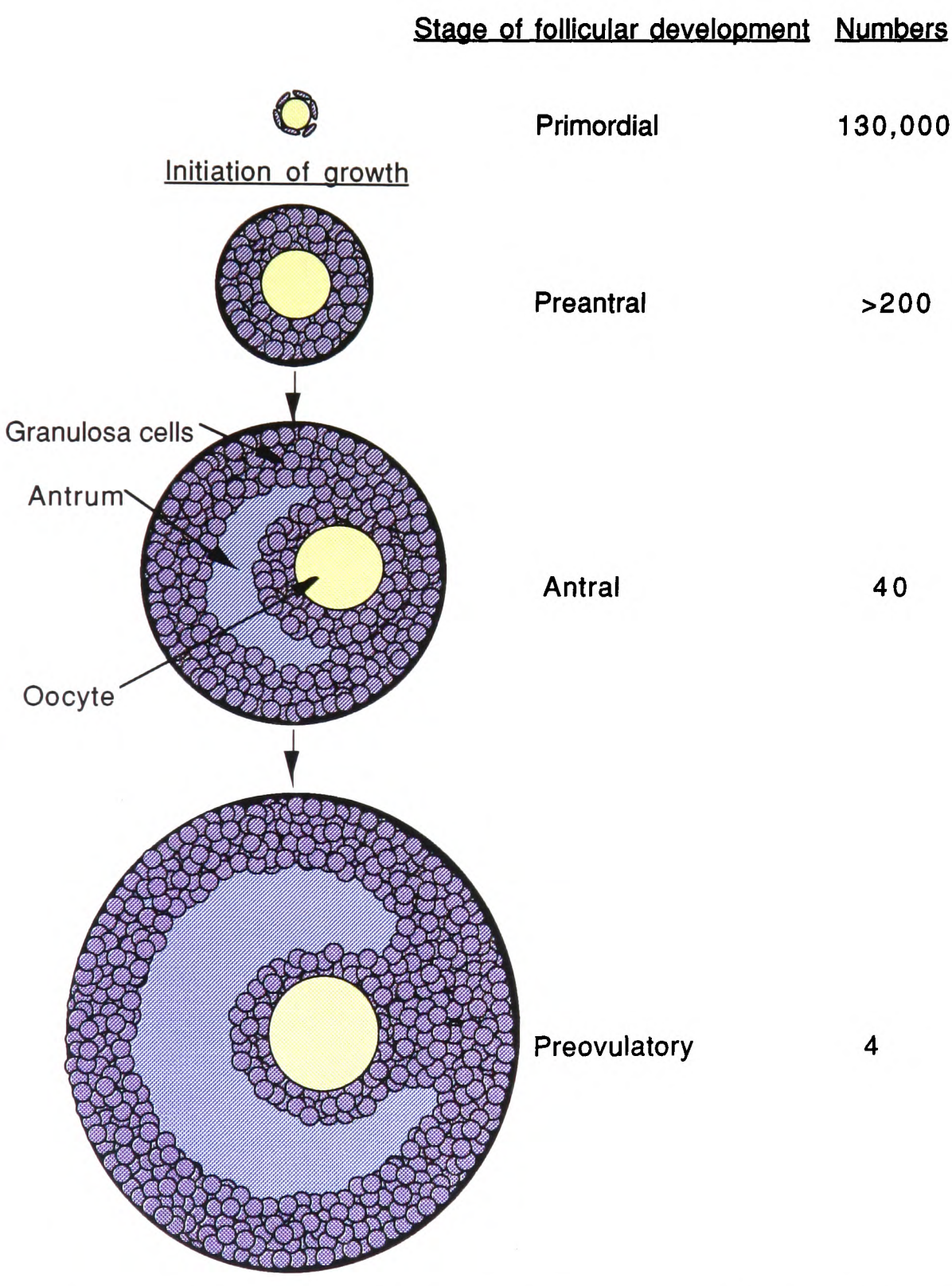


Figure 1.1. The average number of follicles at each stage of follicular development in the post-natal bovine ovary. Adapted from Erickson, 1966.

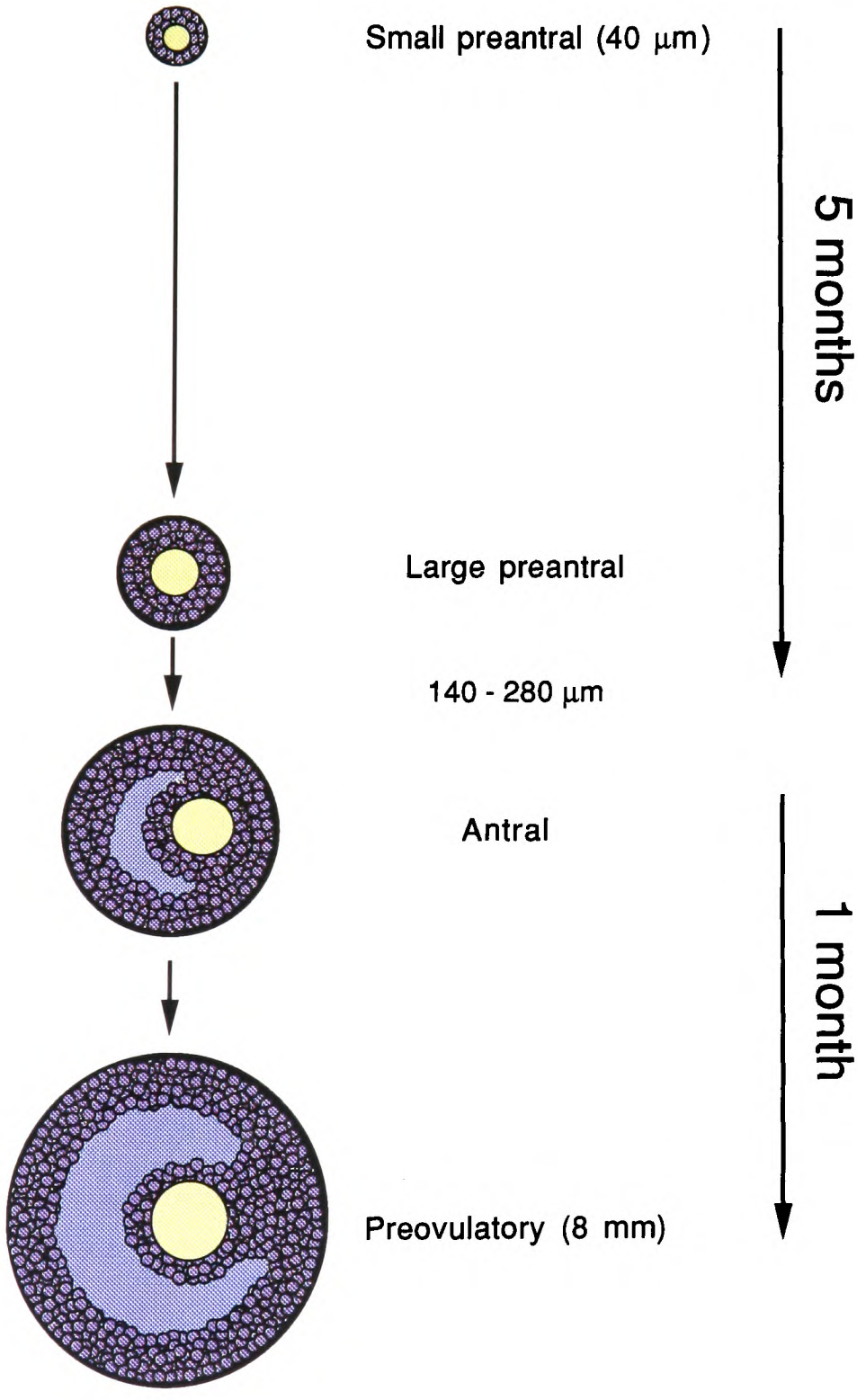


Figure 1.2. Diagram illustrating the stages of bovine follicle development, the approximate sizes of follicles and the duration of each stage. Adapted from Lussier et al., 1987, Monniaux et al., 1984, Figueiredo et al., 1983.

1.1.2. Growing follicles

1.1.2.1. Morphology of follicular development

1.1.2.1.1. Preantral follicles

From the initiation of follicular development to the end of the preantral stage is a lengthy process, taking 4-5 months in cattle (Lussier et al., 1987) (figure 1.2.). During preantral follicle development, the oocyte increases in size and the surrounding granulosa cells become cuboidal (Anderson, 1979). Development of gap junctions between granulosa cells and with the oocyte serves to metabolically couple all cells within the follicle (Anderson and Albertini, 1976, Heller et al., 1981). Outwith the basement membrane, (pre)theca cells contacting the basement membrane can be distinguished from the ovarian stroma by their higher rates of proliferation (Hirshfield, 1991). The theca is vascularised, exposing the follicle directly to circulating factors in the blood (Gosden et al., 1988). There is much debate as to the origin of theca cells and it is unclear whether they are recruited from the stromal cell population or are a different subtype of cells from the start of follicle growth (Hirshfield, 1991). Granulosa cells at this stage are highly proliferative and as the follicle progresses to a multilaminar structure, small pockets of extracellular fluid accumulate within the granulosa cell layers (Gosden et al., 1988). With continuing development of the follicle, these coalesce to form a large cavity, the antrum (Gosden et al., 1988). In cattle, the antrum forms at follicle diameters between 110 μm (10 % of follicles have an antrum) to 280 μm (90 % of follicles possess an antrum) (Monniaux et al., 1984) (figure 1.2.).

1.1.2.1.2. Antral follicle development

This is a comparatively short period of the life span of the follicle, taking between 1-2 months in cattle (Lussier et al., 1987) (figure 1.2.). It is characterised by a rapid increase in follicle size primarily due to follicular fluid accumulation (Lussier et

al., 1987) and further differentiation of the granulosa cells into 2 distinct populations; the mural and cumulus granulosa cells (reviewed by Gougeon, 1996). During the final stages of antrum development, the follicle is acutely sensitive to its hormonal environment and is a major site of steroid production (Gougeon, 1996).

1.1.2.1.3. Antrum formation

The mechanism of antrum formation is not well understood (Gosden et al., 1988) and is further complicated by species differences. In mice, theca cells, which are well differentiated from their stromal counterparts at this stage, may play a role in antrum formation (Torrance et al., 1989, Telfer et al., 1990), whereas in porcine follicles, it appears that structural support may be more important (Hirao et al., 1994).

The function of the antrum is unclear and it has been shown that developmentally competent oocytes can be obtained in vitro without development of the antrum (Eppig and Schroeder, 1989, Carroll et al., 1990). However, the avascular nature of the intact follicle dictates that provision and removal of metabolites must be carried out through diffusion or active transport by follicle cells (Gosden et al., 1988). The antrum is likely to serve as both a pool of nutrients and a dumping ground for waste products. In addition it may also act as a buffer, preventing rapid changes in conditions which may affect the oocyte (Gosden et al., 1988).

1.1.2.1.4. Granulosa cell differentiation

Distinct subpopulations of granulosa cells become evident in the antral follicle (Lederer et al., 1995). Cumulus cell immediately surrounding the oocyte, remain cuboidal and highly proliferative during antrum development and, in the fully developed follicle (Hirshfield and Midgley, 1978), respond to FSH stimulation by secreting hyaluronic acid (Eppig, 1979b). Mural granulosa cells on the other hand are larger, more steroidogenically active, display high aromatase activity and express LH receptors. Granulosa cells originate from a small number of homogenous cells in the

primordial follicle (Telfer et al., 1988) and the factors involved in their differentiation during follicle growth have yet to be identified.

1.1.2.1.5. Morphology of oocyte development

Superimposed on development of the follicle is the preparation of the oocyte for subsequent development after fertilisation. The state of oocyte development in relation to follicle development appears to depend on the species studied. It is of interest that in a number of polyovular species, oocytes have almost reached full size by the end of preantral follicle growth (mouse: Sorenesen and Waasarman, 1976, pig: Hirao et al., 1994), whereas in monovular species, such as cattle, oocytes are less well developed at a comparative follicle stage (Fair et al., 1995b).

Throughout normal oocyte development, meiotic arrest is maintained, with the most obvious changes being in the size of the oocyte. Ultrastructural studies have also shown increases in organelles such as mitochondria, golgi complexes and cortical granules and development of gap junctional communication between the oocyte and surrounding granulosa cells as the oocyte develops (Fair et al., 1995a). Oocytes sequentially acquire meiotic and developmental competence and this appears to be related to oocyte size (Fair et al., 1995b). In cattle, as oocytes reach around 100 μm in diameter, they become meiotically competent but only have a limited ability to sustain embryonic development (Fair et al., 1995b). Progression to diameters greater than 110 μm results in increasing rates of further development (Fair et al., 1995b).

1.1.2.1.6. Follicular demise

The normal fate of follicles is to become atretic with only a very small number reaching ovulation (Hirshfield, 1989). Morphological alterations during follicular atresia have been studied in detail by a number of groups (reviewed by Byskov, 1979, cattle: Marion et al., 1968). Recently it has been discovered that apoptosis is the mechanism underlying follicular atresia in antral follicles at least (Tilly

et al., 1991) and it can be detected before any morphological signs of atresia (Jolly et al., 1994). The role of apoptosis in atresia of smaller follicles is unknown.

1.2. FACTORS AFFECTING FOLLICLE AND OOCYTE DEVELOPMENT

The progression of follicle development is regulated by a large number of factors having endocrine, paracrine or autocrine actions (for review see Gougeon, 1996).

1.2.1. The role of the follicle in oocyte development

The main function of the follicle is to support the growth of the oocyte during its preparation for the resumption of meiosis and subsequent embryonic development. The oocyte is a large cell and its volume to surface area ratio rises with its growth making the supply of adequate nutrients (e.g. pyruvate) and removal of metabolic waste increasingly difficult (Eppig, 1977). Metabolic coupling of the oocyte with the granulosa cells through functional gap junctions makes a larger area of cell membrane available for the uptake of nutrients from the surrounding area (Heller et al., 1981).

Granulosa cells also provide oocytes with metabolites necessary for their normal development. 85 % of the metabolites in the oocyte are of granulosa cell origin (Heller et al., 1981). Removal of granulosa cells results in an alteration of the synthesis and composition of oocyte metabolites (Heller et al., 1981, Crosby et al., 1981) and oocyte growth is suppressed (Heller et al., 1981, Canipari et al., 1984).

Granulosa cells prevent the spontaneous resumption of meiosis. Coincidental with an increase in oocyte size is the acquisition of meiotic competence. The release of meiotically competent oocytes from their surrounding granulosa cells results in spontaneous germinal vesicle breakdown (Eppig and Downs, 1987), indicating an inhibitory role of granulosa cells on meiotic resumption. A granulosa derived factor, termed the maturation inhibiting factor, is thought to be responsible for

the maintenance of meiotic arrest in meiotically competent oocytes (Eppig and Downs, 1984). Little is known about the factor, although steroids and cAMP have been implicated in its regulation (Eppig and Downs, 1984). In addition, its effect is likely to be mediated via gap junctional communication, rather than local secretion. This is further supported by several lines of evidence: in vitro grown denuded oocytes spontaneously resume meiosis and withdrawal of hypoxanthine from cultures of granulosa cell - oocyte complexes, which promotes granulosa cell oocyte association, results in spontaneous germinal vesicle breakdown (Eppig and Downs, 1987).

Gap junctional association of granulosa cells and the oocyte is not necessary for the acquisition of meiotic competence. The culture of initially meiotically incompetent denuded oocytes in either co-culture with somatic cells or their conditioned medium resulted in meiotic resumption corresponding to the timing of acquisition of meiotic competence in vivo despite the lack of oocyte growth (Canipari et al., 1984). It therefore appears that oocyte growth and acquisition of meiotic competence are separable events and that the ability to resume meiosis follows, in part at least, a pre-determined sequence of events initiated at the start of oocyte growth (Canipari et al., 1984). However, denuded oocytes still required a somatic cell produced factor(s) for the acquisition of meiotic competence (Canipari et al., 1984) and cAMP has been identified as one possible factor (Carroll et al., 1991a, Chesnel et al., 1994).

Resumption of meiosis in denuded oocytes grown on a monolayer of somatic cells is arrested at metaphase I (Canipari et al., 1984). Granulosa cell contact during oocyte development is necessary for oocyte growth and acquisition of developmental competence (Heller et al., 1981, Crosby et al., 1981, Canipari et al., 1984). The role of granulosa cells in oocyte growth is unlikely to be purely an enhanced provision of metabolites. Identification of a granulosa produced factor, c-kit, which stimulates oocyte growth (Packer et al., 1994), may be one of many such

factors involved in normal oocyte growth and acquisition of developmental competence.

1.2.3. The role of the oocyte in follicle development

It is increasingly being recognised that the oocyte may also play a role in modulating its follicular environment. It has been proposed that the oocyte, either by direct contact or through the secretion of paracrine factors may influence the acquisition of differentiated function of granulosa cells.

The production of an oocyte secreted factor necessary for FSH stimulated murine cumulus cell expansion has been the most extensively studied oocyte produced factor to date (Buccione et al., 1990a, 1990b, Salustri et al., 1990b, Vanderhyden et al., 1990). Although the factor has yet to be identified, it is known to be heat labile, sensitive to proteinase K digestion and has a molecular weight between 100 - 300 kd, suggesting that it may be a protein or depend on one for its activity (Eppig et al., 1993a). Its secretion is also developmentally regulated (Vanderhyden et al., 1990), coinciding with the acquisition of meiotic competence (Vanderhyden et al., 1990, Eppig et al., 1993b). The oocyte also affects cumulus cell expansion in other ways. Differentiation of granulosa cells into cumulus cells which respond to the cumulus cell expansion enabling factor requires close oocyte contact (Vanderhyden et al., 1990), and after exposure to the factor, another oocyte secreted factor appears to be necessary for cumulus cells to remain responsive to FSH and produce hyaluronic acid (Tirone et al., 1993).

Cumulus expansion enabling factor production has also been shown in other species (pig: Singh et al., 1993, cow: Ralph et al., 1995a), although it is not required for cumulus cell expansion in these species (Prochazka et al., 1991, Singh et al., 1993, Ralph et al., 1995a). Thus, the role of the oocyte in cumulus cell expansion

differs between species and it is likely that other role(s) of the oocyte or its factors between species may be slightly different.

In addition to the role of the oocyte in cumulus cell expansion, it also appears to be involved in the regulation of follicle organisation (Vanderhyden et al., 1990), granulosa cell proliferation (Vanderhyden et al., 1990, 1992) and granulosa cell steroidogenesis (Vanderhyden et al., 1993, Vanderhyden and Tonary, 1995).

The nature of the oocyte secreted factors affecting follicular development are unknown. However, the identification of one factor, GDF-9 (Dong et al., 1996), an oocyte secreted factor involved in granulosa cell development, is significant progress in this direction.

1.2.4. The role of gonadotrophins and steroids in follicular development

The primary role of the gonadotrophins is to initiate and sustain follicular functions (Gougeon, 1996). This study is concerned with the development of preantral and early antral follicles, generally thought to be unresponsive to gonadotrophin stimulation. However, there is a substantial body of evidence gathering which shows that FSH plays a role in stimulating granulosa cell proliferation and differentiation during early follicular development (hamster: Chiras and Greenwald, 1978, Roy and Greenwald, 1987), although it is not essential for their survival (rat: Nakano et al., 1975). In contrast, as the antrum develops, the follicle becomes increasingly dependent on gonadotrophins for continued development (Hirshfield and Midgley, 1978. Halpin et al., 1986).

The role of FSH and LH in follicular steroid production by the classical 2 cell, 2 gonadotrophin mechanism is now well established (Armstrong et al., 1979). Briefly, cholesterol is converted to androgens in the theca cells by a number of enzymes which are under the control of LH (Armstrong et al., 1979). These androgens can then be utilised by the granulosa cells which convert them to oestrogens

(principally oestradiol 17β) by the p450 aromatase enzyme system (Armstrong et al., 1979). The aromatase enzyme system requires the activation of a cAMP signal transduction system which is initiated by the binding of FSH to its surface receptors on the granulosa cells (Armstrong et al., 1979), the exclusive site of FSH receptors in the follicle (Midgley, 1973).

The smallest preantral follicles have been shown to respond to FSH and produce steroids (hamster: Roy and Greenwald, 1987). However, it is only during the later stages of preantral follicle development that the theca cells of these follicles acquire the ability to respond to LH through expression of functional receptors on the theca cells (hamster: Roy and Greenwald, 1987), to produce aromatisable androgens. Once their receptors are expressed, LH appears to be beneficial to the growth of follicles from the large preantral stage onwards (Spears et al., 1994).

The precise nature of the gonadotrophin regulated, granulosa-theca cell interaction during follicular steroidogenesis is likely to vary between species (Ryan, 1979) and within species (Fry and Draincourt, 1996). Much of the early work examining this interaction was conducted in laboratory rodents (hamster: Roy and Greenwald, 1987, rat: Wolff-Exalto, 1982, mice: Halpin and Charlton 1988) and comparatively little is known about larger mammals. For example, the inability to localise aromatase in granulosa cells of bovine follicles < 3 mm is of interest (Lautinick et al., 1994) and requires further investigation.

Oestrogens stimulate granulosa cell mitosis, differentiation by the induction of FSH (and later LH) receptors (Hillier, 1987) and prevention of atresia (Wang and Greenwald, 1993). Their actions amplify the influence of the gonadotrophins on follicular development, making the follicle more sensitive to gonadotrophin stimulation and may also play a role in follicle selection (Hillier, 1987). There is also evidence of bi-directional communication between the granulosa and theca cells in the regulation of steroidogenesis (Hillier et al., 1994). Thecal cell androgens binding to their receptors

on the granulosa cells may modulate the cells responsiveness to FSH (Hillier et al., 1994) and a granulosa cell factor (possibly inhibin) may affect thecal androgen production (Hillier et al., 1994).

Our poor understanding of the effects of gonadotrophins on follicular development are exemplified by the fact that progress in improving the reliability of superovulation in cattle using gonadotrophins has been slow (Hasler, 1992). Follicle cell produced factors modulate the effects of gonadotrophins on follicular development and an appreciation of their action may enable a better understanding of the role of gonadotrophins in follicular development.

1.2.5. The role of granulosa and theca cell produced factors on follicular development

A number of factors are produced by the granulosa cells which have actions within the follicle. The main groups of granulosa cell produced factors are the inhibin related peptides and growth factors.

1.2.5.1. Inhibin related peptides

The inhibin related peptides (inhibin, activin, follistatin and TGF-β, have been shown to have both endocrine and paracrine/autocrine actions. Their production is regulated by a complex interaction involving the gonadotrophins, growth factors (EGF, TGFα, TGFβ, IGF-I) and oestradiol (Findlay, 1993, Aloi et al., 1995). In addition, recent evidence also implicates a role for the oocyte in the synthesis of inhibin, activin and TGFβ (Braw-Tal, 1994).

1.2.5.1.1. Inhibin

The regulatory role of inhibin in depressing FSH secretion by the anterior pituitary has been well documented (Farnworth et al., 1988, Tonetta and diZerega, 1989). It is thought that the ability of dominant follicles to secrete large amounts of

inhibin, reducing peripheral FSH, restricts the growth of non-dominant follicles (Findlay, 1993). In rats, this action is augmented by the paracrine action of inhibin, stimulating thecal androgen production, the substrate for oestradiol production by the granulosa cells (Hillier et al., 1991). However, recent studies have not detected a similar mechanism in bovine follicles, suggesting that some species differences exist in the actions of inhibin (Shukovski et al., 1993).

1.2.5.1.2. Activin

Measurement of activin activity, being a homodimer of β subunits shared with inhibin and TGFβ, has proved difficult (Findlay 1993). Nevertheless, it has been shown to stimulate the secretion of FSH from the anterior pituitary (Ling et al., 1988). Of particular relevance to the stages of follicles which will be examined in this project, is the role of activin in the differentiation and acquisition of FSH responsiveness of granulosa cells from large preantral and early antral follicles (Findlay, 1993) by stimulating the appearance of FSH receptors (Findlay, 1993, Nakamura et al., 1995). In addition, activin also stimulates rat granulosa cell proliferation (Miró and Hillier, 1996, Li et al., 1995) and in combination with FSH, appears to be involved in follicle organisation and antrum formation (Li et al., 1995). However these studies used either cultures of isolated granulosa cells (Miró and Hillier, 1996) or follicle cultures in which the granulosa cells were allowed to form monolayers (Li et al., 1995) and further evidence of the effects of activin on intact preantral and early antral follicles is required.

1.2.5.1.3. Follistatin

In contrast to inhibin and activin, sharing a common β subunit, follistatin is a single peptide chain. The presence of follistatin has been detected in early preantral follicles and its levels generally increase as the follicle develops (Braw-Tal, 1994, Findlay, 1993). Its synthesis is stimulated by both FSH or activin (Klein et al., 1991,

Findlay, 1993) and its primary effects are the suppression of FSH release (Findlay, 1993) and actions antagonistic to those of activin for which it is a binding protein (Nakamura et al., 1990).

1.2.5.2. Growth factors

A number of growth factors produced by the granulosa and theca cells are involved in the modulation of folliculogenesis, the effects of which depend on a complex interaction between other growth factors and hormones. Ovarian growth factors, their regulation and actions have been excellently reviewed elsewhere (Tonetta and diZerega, 1989, Guidice, 1992, Carson et al., 1989, Monget and Monniaux, 1995). The most important growth factors relevant to follicular development will be described briefly here.

1.2.5.2.1. IGF

The IGF system comprises peptides (IGF-I, IGF-II), specific receptors on target cells and a number of binding proteins which regulate bioavailability (Guidice, 1992, Samaras et al., 1993). The precise actions of the IGFs depend on the species and the interaction of gonadotrophins (Guidice, 1992). However, in general they increase granulosa cell proliferation, aromatase activity and oestradiol production (Guidice, 1992, Tonetta and diZerega, 1989). The effects of IGFs during preantral follicle development are likely to be limited, in cattle at least, as receptors on granulosa cells are low in number until after development of the antrum (Wandji et al., 1992b).

1.2.5.2.2. EGF

In the bovine follicle, EGF receptors appear on the granulosa cells of large preantral follicles (Wandji et al., 1992b). The actions of EGF are thought to mediate some of the effects of FSH (hamster: Roy and Harris, 1994). From in vitro studies of whole follicles, EGF has been shown to reduce steroid production (mouse: Boland

and Gosden, 1994) and stimulate granulosa cell proliferation (cow: Wandji et al., 1996a), although the latter effect may also be due to an interaction with TGFβ (Roy, 1993).

1.2.5.2.3. TGFβ

The effects of TGFβ vary depending on the species studied (Tonetta and diZerega, 1989). In bovine preantral follicles, TGFβ has been shown to inhibit follicle growth and antagonise FSH stimulated granulosa cell differentiation and steroid production (Wandji et al., 1996a).

1.2.5.2.4. bFGF

Receptors to bFGF are present on the granulosa cells of preantral bovine follicles (Wandji et al., 1992b). The action of bFGF appears to keep granulosa cells in preantral follicles mitotically active and maintains their relatively undifferentiated state by inhibiting FSH induction of LH receptors (Mondschein and Schomberg, 1981), oestradiol and progesterone synthesis (Wandji et al., 1996a). In addition, bFGF may also affect oocyte development, although the absence of bFGF receptors on the oocyte (Wordinger et al., 1993) suggests that this may be an indirect effect via the granulosa cells.

1.3. EXAMINING FOLLICULAR DEVELOPMENT

1.3.1. Studies of follicular development using in vivo models

Techniques such as ultrasonography (cattle: Bergfeld et al., 1994) have provided a great deal of information on the later stages of follicular development. However, these techniques are not sensitive enough to detect changes in preantral or early antral follicles.

Removal of the ovary and sectioning for histological analysis has enabled studies of the general morphology of ovarian follicles (cattle: Marion et al., 1968).

Pretreatment of the animal before ovariectomy, by the administration of hormones (e.g. hamster: Chiras and Greenwald, 1978) or using techniques such as hypophysectomy (e.g. rat: Nakano et al., 1975) have given insights into the roles of factors such as gonadotrophins in early follicular development. However, in vivo mechanisms counteracting the effects of these treatments may confound analysis. In addition, these systema are restricted to analysis at a single time point and does not lend itself to following the progression of follicular development.

1.3.2. Studies of follicular development using in vitro models

1.3.2.1. Granulosa cell cultures

Isolation and examination of granulosa cells has provided a useful tool for detecting the effects of growth factors and hormones on their development in a number of species (rat: Mondschein and Schomberg, 1981, pig: Buck and Schomberg, 1987, cow: Savion et al., 1981, Gong et al., 1993). However, it must be remembered that the main function of the follicle is to support the development of the oocyte and, as discussed earlier (chapter 1.3.3.), the oocyte plays a critical role directing granulosa cell function. Extrapolation of the findings from isolated granulosa cell cultures to the in vivo situation must therefore be made with care.

1.3.2.2. Whole ovary culture

Whole ovaries can be maintained in vitro for up to 4 days using a perfusion system (mouse: Romanoff and Pincus, 1962, Ryle, 1969, 1971). A number of groups have used such systems to examine the effects of factors such as gonadotrophins on follicle development (Ryle 1969, 1971) and ovulation (Bränström and Flaherty, 1995). However, following the development of the smaller follicles during culture within a large intact ovary will be difficult and as a result, the findings based on this system are restricted to the final stages of follicular development.

1.3.2.3. Follicle culture

Early studies of the isolation and culture of granulosa cell enclosed oocytes from large antral murine follicles, their maturation and fertilisation in vitro (Pincus and Enzman, 1935) demonstrated the possibility of increasing the number of oocytes obtained per ovary and also the use of culture to examine factors affecting follicular development. Studies such as these formed the basis of in vitro maturation and fertilisation systems in use today. However, it was not until more recently that the possibility of using the much larger pool of oocytes within preantral follicles, by developing methods for their growth in vitro, was recognised (Roy and Greenwald, 1986, Torrance et al., 1989, Eppig and Schroeder, 1989). Subsequent improvements resulted in the production of developmentally competent murine oocytes following culture of isolated preantral follicles (Eppig and Schroeder, 1989). Since then, numerous studies have attempted to improve this work further or apply it to other species, particularly those of commercial value such as cattle.

Current methods for the growth of oocytes in vitro can be broadly classified into 2 types based on the reason of study: (i) those which are primarily for the production of large numbers of viable oocytes or studying oocyte development or (ii) those used as models for examining follicular development.

1.3.2.3.1. In vitro growth of follicles for oocyte production or examining oocyte development

The common feature of these systems is the employment of an enzymatic isolation procedure (Nicosia et al., 1975) which rapidly releases large numbers of preantral follicles. However, during isolation, theca cells are lost and degradation of the basement membrane occurs leaving an oocyte surrounded by several layers of granulosa cells (a granulosa cell oocyte complex (GOC)) (Eppig et al, 1996). Despite the use of this system to examine a number of factors affecting follicular development (Roy and Greenwald, 1989, Wang et al., 1991, Torrance et al., 1989, Eppig et al.,

1992), the absence of a theca cell layer and an intact basement membrane limits its usefulness for examining a number of aspects (e.g. steroidogenesis, antrum formation). Nevertheless, during culture the oocytes grow and acquire developmental competence which has been verified by the production of live offspring (Eppig and Schroeder, 1989). A key component to the success of oocyte growth in vitro is the maintenance of the 3 dimensional granulosa cell organisation and contact with the oocyte which is necessary for normal oocyte development (chapter 1.3.1.). Culture systems which permit granulosa cells to become detached from the oocyte have not been successful in producing developmentally competent oocytes (Daniel et al., 1989).

The ease with which large numbers of complexes are released and successfully grown in vitro makes this an attractive system for the production of competent oocytes and for studying oocyte development. To date this system has only been successfully applied to murine species to produce embryos (Eppig and Schroeder, 1989, Spears et al., 1994), although recent studies have indicated that with further improvement its application for porcine preantral follicle growth is possible (Hirao et al., 1994).

1.3.2.3.2. Follicle development systems.

Isolation by microdissection has been used to release preantral follicles with a stromal/theca layer and intact basement membrane (Nayudu and Osborn, 1992). Culture of these follicles have proved to be a useful model for examining interfollicular effects (Nayudu and Osborn, 1992, Spears et al., 1996), steroidogenesis (Nayudu and Osborn, 1992, Boland et al., 1993), metabolism (Boland et al., 1993) and the acquisition of developmental competence during culture (Spears et al., 1994) and has the advantage that the results are more applicable to in vivo follicle development. However, the dissection procedure is time consuming, limiting the use of this system as a large scale method of oocyte production, although it may be more appropriate for preantral follicle isolation from the ovaries of mature and/or larger mammals which are

more resistant to enzymatic digestion. In addition, the size of antral follicles from large mammals may preclude the possibility of continued culture of intact preantral follicles.

1.3.2.3.3. Applications of in vitro oocyte growth

Assisted reproduction technology (e.g. superovulation, IVM, IVF) has a wide range of applications from overcoming human infertility to improving animal production. The major disadvantage of current techniques is that they rely on the small population of large antral follicles and as a result, the success is limited (Monniaux et al., 1983, Boland and Roche, 1993).

In vitro growth of oocytes from preantral follicles overcomes this problem as there are large numbers within the ovary, although their use will require lengthy culture. Nevertheless, the possibility of producing large numbers of developmentally competent oocytes does have clear advantages for the preservation of rare breeds, increasing the number of offspring from animals of high genetic merit, reducing the need for unreliable superstimulatory techniques, providing a large number of homogenous oocytes for use in new reproductive techniques such as nuclear transfer (Campbell et al., 1996) and as a research tool. Murine preantral follicles can be frozen until required and grown in vitro to produce developmentally competent oocytes (Carroll et al., 1990, 1991b). If adapted to other species, this offers a number of exciting possibilities such as restoring fertility to chemotherapy patients by removal of material before treatment or maintaining the gene pool by storing follicles from breeds of cattle which are not ideally suited to current agricultural climate but may be useful in the future. Further advances in increasing the rates of genetic improvement in livestock may also be possible by reduction of the generation interval through the use of in vitro grown oocytes from foetal ovaries (Betteridge et al., 1989).

1.3.2.3.4. Isolation and culture of bovine preantral follicles

Adaptation of techniques used for the successful in vitro growth of murine oocytes from preantral follicles (Eppig and Schroeder, 1989, Carroll et al., 1991, Spears et al., 1994) have yet to reproduce similar achievements in other species. Differences in the ease of isolation, sensitivity to collagenase and the length of culture required for substantial growth (Lussier et al., 1987) have proved to be initial problems for bovine preantral follicle culture.

In cattle considerable attention has been focused on the isolation and culture of early preantral follicles (Jewgenow and Pitra, 1991, Figueiredo et al., 1994, Nuttinck et al., 1993). Prolonged culture will be required if developmental competent oocytes are to be obtained from these follicles, limiting the current usefulness of this approach as a research tool. Isolation of more developed large preantral follicles about to enter the rapid phase of antral growth, although smaller in number, may circumvent this particular problem. Successful in vitro growth of these follicles will require development of a suitable isolation procedure and culture conditions identified as necessary to sustain follicular development.

AIMS

Our understanding of the mechanisms governing follicular development are poor. Two areas in which current knowledge is particularly defficient are: (i) the control of early follicular development and (ii) the role of the oocyte in follicular development. The aims of this study are therefore:

(i) To develop a model for studying preantral follicle development

(ii) To examine the effects of factors on preantral follicle development

(iii) To examine the role of apoptosis in preantral follicle development

(iv) To examine potential roles of the oocyte in follicular development.

The species of study will be cattle in which an increased awareness of the regulation of follicular development will have important implications for animal production and modelling of human infertility.

CHAPTER 2. GENERAL METHODS

2.1 FOLLICLE ISOLATION

2.1.1. Dissection medium

Dissection medium was used for all bench manipulation of ovarian material. It was prepared by supplementing Earle's TCM199 10X (cat.no. 21180-013, Life Technologies, Paisley, UK) (diluted to 1X concentration by addition of appropriate amount of sterile distilled water) with 7.08 mg/ml hepes (Sigma, Poole, Dorset, UK), 75 μg/ml kanamycin monosulphate (Sigma) or 50 μg/ml gentamicin (Life Technologies). The pH, measured by a pH meter (model 240, Corning Suffolk, UK), was adjusted to pH7.4 by the addition of 1M sodium hydroxide (Sigma). Osmolarity, measured by an osmometer (model 3MO plus, Advanced Instruments, Ma, USA), was adjusted to 279 mOsm/kgH_2O by addition of sterile distilled water. The medium was filtered (0.22 μm membrane, Corning, NY, USA) by suction into a sterile bottle and stored at -4 ^oC until use for a maximum of 2 weeks after preparation. As required, aliquots of the medium were warmed to 39 ^oC in an incubator (Flow Laboratories, Irvine, UK) and supplemented with 10 % heat inactivated foetal calf serum (FCS) (Globepharm, Surrey, UK). The FCS did not contain detectable levels of FSH or LH, IGF-I at a concentration of 60 ng/ml was present (determined using an ELISA immunoenzymatic assay kit (Reprokit, Sanofe Sante Animale, Libourne Cedex, France), sensitivity for LH and FSH = 0.5mIU/ml, performed by the department of Clinical Chemistry, Royal Infirmary, Glasgow as part of their routine bovine serum studies). The FCS was previously heat inactivated by heating at 56^oC for 15 minutes, aliquoted and stored at -20 ^oC until required.

2.1.2. Collection of cortical slices

Bovine ovaries were obtained from an abattoir at 25-30 ^{o}C, rinsed with industrial methylated spirits and then soaked in PBS (Unipath Limited, Basingstoke, UK) with 50 μg/ml gentamicin (Life Technologies). All subsequent manipulation of the ovarian material was conducted in a laminar flow hood (Gelaire, Flow Laboratories). Fine slices of the ovarian cortex were taken using a scalpel and placed in dissection medium

2.1.3. Micro-dissection

Under a dissecting microscope (Wild M3Z, Heerbrug, Switzerland), fitted with base illumination, an adjustible mirror, a 39 ^{o}C heated stage (P. Miles, Longstanton, Cambridgeshire, UK) and a calibrated eyepiece graticule (Graticules Limited, Tonbridge, Kent, UK), bovine large preantral/early antral follicles were dissected from the cortical slices using fine 25 G needles (Terumo Europe, Leuven, Belguim) attached to syringe barrels. Dissection was carried out in a large (85 mm) plastic petri dish (Bibby Sterilin, Stone, UK) under high illumination to visualise the follicles within the cortical slices. Dissected follicles were moved to a small (45 mm) petri dish (Bibby Sterilin) containing fresh dissection medium using a pipette.

2.1.4. Follicle classification

Isolated follicles of approximately 90 to 270 μm in diameter with several stromal/theca cell layers, an intact basement membrane, evenly pigmented granulosa cell layer and oocyte were selected for culture. Selected follicles were divided into 3 size classes: preantral (approximately 90 μm to 150 μm), large preantral/early antral (approximately 150 μm to 210 μm) and antral (approximately 210 μm to 270 μm). Follicles were held in dissection medium until the start of culture. The entire process

from collection of material from the abattoir to the start of culture was always less than 7 hours.

2.2. FOLLICLE CULTURE

2.2.1. Extraction of collagen from rats tails

Rat tail collagen was prepared by the method described by (Chambard et al., 1981, Eppig and Telfer, 1993). Briefly, frozen rat tails were thawed in 70 % ethanol overnight. In a laminar flow hood, the tendons were extracted by breaking sections of the tails with a pair of forceps and pulling out the strands of collagen. The collagen strands were washed in sterile distilled water and blotted dry with filter paper. 100 ml of a 0.1% acetic acid in sterile distilled water solution was added for every 1 g of collagen strands. The mixture was covered, left to stir for 48 hours at <4 oC and then centrifuged in 50 ml centrifuge tubes at 4,000 rpm for 60 minutes at <4 ^{o}C. The supernatant was withdrawn and aliquoted into fresh tubes. Batches of the collagen were stored at - 20 ^{o}C until required. During use, aliquots were stored in a 4 ^{o}C until required for culture.

2.2.2. Culture medium

Culture medium was prepared by supplementing TCM199 (Sigma) with 50 µg/ml gentamycin (Life Technologies), 250 µg/ml sodium pyruvate (Sigma), 50 µg/ml insulin (Sigma), 10 µg/ml transferrin (Sigma). The medium was filtered and stored as described for dissection medium above. As required, the medium was warmed to 39 ^{o}C in an incubator for at least an hour before the start of culture. The top of the flask was left open to allow the pH of the medium to adjust in the 5 % CO_2 atmosphere. The medium was supplemented10 % FCS (Globepharm) before use.

2.2.3. Collagen gel coating of wells

The collagen gel coating of the wells has been described previously (Eppig and Telfer 1993). In summary, the collagen was prepared on ice by mixing 1.6 ml of rats tail collagen, 200 µl of M199 10X (cat. no. 21180-013 Life Technologies), 200 µl sterile PBS and 40 µl sodium hydroxide (Sigma). 4 well tissue culture plates (Nunc, Roskilde, Denmark) were coated with 200 µl of collagen mix and allowed to set at 39 ^{o}C. 1 ml of culture medium was used to wash the collagen. Culture wells were incubated at 39 ^{o}C, 5 % CO_2 in a sterile, humidified incubator (Biocenter 2001, Salvis AG, Reussbühl, Switzerland) for at least one hour before the initiation of culture. Fresh medium was added before the start of culture.

2.2.4. Initiation of culture

4 follicles were added to each well and evenly spaced to allow identification of individual follicles throughout culture. Follicle and oocyte diameters were measured using an inverted microscope (Nikon Diaphot, Nikon, Japan) fitted with a calibrated, crossed micrometer (Graticules Limited, Tonbridge, Kent, UK). Follicle diameter measurements were taken in perpendicular planes. The average follicle diameter was calculated and used for analysis.

2.2.5. Measurements and medium changes during culture

Half the culture medium was changed on day 1 of culture and every second day thereafter. At the same time as medium changes, follicle and oocyte measurements were made as described above. Follicles becoming degenerate during culture were excluded form the results.

2.3 HISTOLOGY

2.3.1. Fixation

At the end of the culture period, follicles were dislodged from the substrate, washed in fresh medium and fixed overnight in Bouins solution (70% Picric acid, 25% formaldehyde, 5% glacial acetic acid). The fixative was then removed and replaced with 70% alcohol and left until required. Addition of a small amount of Eosin (BDH, Poole, Dorset, UK) to the 70% alcohol aided their visualisation during processing.

2.3.2. Follicles for morphological assessment

2.3.2.1. Processing of samples

Follicles for image analysis or morphological assessment were dehydrated by repeated changes through a series of increasing alcohol concentrations up to absolute alcohol. Absolute alcohol was replaced with cedar wood oil (BDH) for a minimum of 24 hours. When removed from the oil, follicles were cleared in toluene (in a fume hood) for 30 minutes and then placed in plastic wells (cat. no. 18985, Polysciences Inc., Warrington, Pa, USA) containing paraffin wax (Sherwood Medical Company, St. Louis, Mo, USA) in a 60 ^{o}C oven. The parraffin wax was replaced every hour for 4 hours to remove all traces of toluene. After the final change, the follicles were manouvered into the centre of the well with warm forceps and the blocks were cooled in cold water.

2.3.2.2. Sectioning and mounting

The samples were sectioned at 6 μm using a microtome (Leica, model Jung RM2035, Nussloch, Germany). The sections were floated onto gelatin coated slides (Chance Propper Limited, Warley, UK) (prepared by dipping in a solution of 1 % gelatine (Sigma), 0.1 % chromic potassium sulphate (May and Baker Limited,

Dagenham, UK) in distilled water) in a water bath at 42°C and allowed to dry overnight in a 37°C oven.

2.3.2.3. Staining

The sections were dewaxed in xylene for 15 minutes and then taken down through a series of alcohols (absolute to 70 %) leaving them in each concentration until miscible. The staining procedure was conducted as follows (for reagent preparation see Drury and Wallington, 1976): a quick dip in 70 % alcohol with lithium carbonate (to remove Bouins fixative), rinse in water, 5 minutes in Harris' Haemotoxylin, rinse in running water until it becomes colourless, 2 dips in acid alcohol, rinse in water, 3 minutes in Scott's tap water substitute, wash in water, 2 minutes in Eosin (1:1 working solution), dip in tap water, 3 minutes in potassium alum (BDH), rinse in tap water. The sections are then taken up a series of graded alcohols to absolute alcohol and then left in xylene until it becomes clear. The sections can then be covered in DPX mounting medium (BDH) and a glass coverslip.

2.3.3. Processing of follicles for autoradiography

2.3.3.1. Tritiated thymidine labelling of follicles

0.185MBq of tritiated thymidine (Amersham Life Science, Buckinghamshire, UK.) was added to each culture well during the last 24 hours of culture. At the end of culture, follicles were washed twice in fresh medium before fixing. Sections of follicles were obtained as described above and mounted on uncoated, washed glass slides.

2.3.3.2. Autoradiography

In the dark room, the slides were dipped in a 1:1 mix of photographic emulsion (Ilford K-5, Ilford Ltd., Mobberley, Cheshire, UK.) and distilled water which had been heated in a water bath at 43 °C. To ensure even distribution of

emulsion the slides were allowed to dry overnight in a large light proof box and then placed in exposure boxes with a small amount of silical gel. The slides were exposed for a total of 3.5 days at room temperature.

2.3.3.3. Developing and staining sections

Slides were developed in the dark room by dipping in developer (Kodak D-19, Kodak-Pathe, Paris, France) for 2 minutes, rinsed in 1 % acetic acid for 1 minute, fixed in a 30 % solution of sodium thiosulphate and then washed in tap water for 20 minutes. Following developing, the sections were lightly stained with haemotoxylin and eosin as follows: 3 minute dip in Harris's haemotoxylin, wash in running water until it becomes clean, 3 quick dips in acid alcohol (70 % ethanol, 1 % conc. HCl), 3 quick dips in water followed by 15 seconds in eosin. The slides were then taken up through a graded series of alcohols and mounted as described above.

2.3.4. Semi-thin sections for detailed examination of follicle morphology

Follicles were fixed in Bouins as described above and then stored in 70 % alcohol. Follicles were embedded in plastic resin (LR White resin (medium), TAAB Laboratory Equipment Ltd., Reading, UK.) in small beam capsules (Agar, Stansted, UK) and sectioned at 2 μm using a ultramicrotome (Leica). Every fifth section was mounted by dropping the section onto a heated, washed glass slide. The sections were covered with Toluidine blue and heated for 15 seconds on a hot plate (60 ^{o}C) and then rinsed with water.

2.3.5. Collection of results

Histological observations and measurements were made under the light microscope (Nikon microphot, Nikon) fitted with a crossed micrometer (Graticules Limited). The section containing containing the oocyte nucleolus, or largest cross

section of the oocyte if the nucleolus was absent, was used for observations and measurements.

CHAPTER 3: ISOLATION OF BOVINE PREANTRAL FOLLICLES

3.1. INTRODUCTION

Isolation and culture of individual follicles provides a convenient model to examine follicular development and provide the possibility of utilising the large pool of immature follicles to increase oocyte production. Initial studies of preantral follicle isolation and culture have concentrated on murine species (for reviews see: Gosden et al, 1993, Eppig et al., 1996). Recently, a number of groups have attempted to adapt systems developed in murine species to larger mammals (for review see: Telfer, 1996).

Whether murine follicles are to be cultured for oocyte production or examining follicular development determines the method of isolation: 1. In murine species, rapid enzymatic isolation techniques have been developed which release large numbers of follicles (Roy and Greenwald, 1985, Eppig and Downs, 1987). However, these methods of isolation remove both thecal cells and the basement membrane leaving a granulosa cell-oocyte complex (GOC) limiting the use of GOC for detailed studies of follicular development. Nevertheless GOC's can be grown in vitro (mouse: Eppig and Schroeder, 1989, hamster: Roy and Greenwald 1989) producing developmentally competent oocytes with a high rate of success (Eppig and Schroeder, 1989) and their usefulness for both studies of oocyte development and assisted reproduction have been demonstrated (for review see: Eppig et al., 1996). 2. Isolation of intact preantral follicles by micro-dissection has been used initially in murine species (Nayudu and Osborn, 1992). In contrast to GOC, the basement

membrane and stromal/thecal tissue are retained in micro-dissected follicles through avoiding the use of enzymes such as collagenase. The isolation process is generally more tedious and time consuming yielding fewer follicles than the GOC isolation method and as a result, limits its potential as a source of oocytes for assisted reproduction. Developmentally competent oocytes have been grown from micro-dissected murine preantral follicles (Spears et al., 1994), although their culture has been used primarily as a model to examine follicular development as it is of greater physiological relevance than the use of GOC.

The direct application of murine preantral follicle isolation methods to cattle ovaries has proved difficult because they are larger, more fibrous and the follicles are less densely packed. Nevertheless, large numbers of small preantral bovine follicles have been isolated from cattle ovaries by mechanical and/or enzymatic methods similar to those devised for hamster preantral follicles by Roy and Greenwald (1985) (Jewgenow and Pitra, 1991, Nuttinck et al., 1993, Figueiredo et al., 1993, Telfer, 1996 (review)). In vitro growth of small preantral follicles to the preovulatory stages will require lengthy culture periods, although studies of their growth in vitro may provide valuable insight into both the physiology and the culture conditions required for early follicular development. An alternative approach would be to isolate preantral follicles as they are about to enter the rapid phase of antral growth. This would necessitate a much shorter period of culture if developmentally competent oocytes are to be obtained. However, due to selection processes within the ovary, the number of large preantral follicles is far fewer than the number of small preantral follicles (Monniaux et al., 1984, Lussier et al., 1987). Isolation of large preantral follicles using enzymatic digestion by collagenase has recently been demonstrated (Wandji et al., 1996a). In common with the isolation of smaller bovine follicles (Nuttinck et al., 1996), a detrimental effect of collagenase on the oocyte was noticed (Wandji et al., 1996a). An enzyme-free method is therefore preferable for the isolation of bovine preantral follicles

Primordial follicles constitute the largest follicle population within the ovary. Recent work showing the initiation of murine primordial follicle growth in vitro to produce competent oocytes (Eppig and O'Brien, 1996) is the result of much effort into devising suitable systems for the growth of murine oocytes in vitro. Initiation of bovine primordial follicle growth can now be achieved in vitro (Wandji,et al., 1996b). Considerable effort is required to devise a suitable system(s) to support complete growth of bovine follicles in vitro. However, development from the early preantral to preovulatory stages takes up to 7 months in vivo (Lussier et al 1987) and is an ambitious aim for culture. It is likely that a combination of culture techniques will be required depending on the stage of follicular development. In this study, different methods of isolating large preantral follicles from mature bovine ovaries were examined so that the conditions required for their growth in vitro could be evaluated. Only micro-dissection of follicles without the use of enzymes provided sufficient numbers of intact follicles of the desired size for future culture experiments.

3.2. METHODS

Bovine ovaries were obtained from an abattoir and maintained at 25-30°C during transit. The ovaries were rinsed with industrial methylated spirits and soaked in PBS with 50mg/ml gentamycin (Life Technologies, Paisley, UK) and kept warm (39°C) using a water bath.

3.2.1. Localisation of preantral follicles within the ovary

Thin (1-2mm) transverse sections of fresh bovine ovaries were taken using a scalpel blade. The sections were fixed overnight in Bouins solution and then transferred to 70% ethanol for at least 24 hours. The sections were then processed through a series of graded ethanol's up to absolute ethanol and left to clear overnight in toluene. The sections were initially treated with a toluene:paraffin wax (1:1) mix for 1 hour in a vacuum oven at 60°C and then transferred to fresh paraffin wax for an additional 4 hours. The samples were sectioned at 6μm using a microtome, mounted on gelatine coated glass slides, stained with haemotoxylin and eosin (as described in chapter 2) and viewed under the microscope.

3.2.2. Follicle Isolation

3.2.2.1. Enzymatic digestion

Fine slices of the ovarian cortex were taken using a scalpel and placed in dissection medium (chapter 2) containing collagenase at concentrations of either 0, 1, 3, 6 and 12mg/ml for 30 minutes at 37°C. After incubation, the slices were placed on a fine tissue filter (pore size = 380μm) (cell dissociation sieve, Sigma) and any digested tissue washed through the sieve with fresh dissection medium. The filtrate was allowed to settle for 20 minutes at 37°C and the overlying medium was drawn off. The pellet was resuspended in fresh dissection medium and poured onto a plastic petri dish (Bibby Sterilin, Staffordshire, UK) with a guidelines drawn on the

underside to aid searching. The petri dish was searched using a dissection microscope fitted with a heated stage. Follicles were collected using a pipette and placed in a micro tube.

3.2.2.2. Enzymatic digestion and homogenisation

Ovarian cortical slices were incubated in dissection medium with 3 mg/ml of collagenase for 30 minutes as described above. The tissue was then ground with glass beads using a mortar and pestle until a homogenate of a smooth consistency was obtained. The homogenate was filtered using a tissue filter (pore size = 360μm) (Sigma), allowed to settle, resuspended in dissection medium and searched under the dissection microscope as described above.

3.2.2.3. Mechanical isolation

Cortical slices were placed in a liquidiser (Osterizer, Oster Co., Wisconsin, U.S.A.) with 10 ml of dissection medium. The contents were liquidised until a mixture of smooth consistency was obtained. The homogenate was placed in a centrifuge tube and centrifuged at 2000 g for 2 minutes, the overlying medium removed, and the residue resuspended in fresh medium. The homogenate was sequentially filtered through a 380 μm, 190 μm and 104 μm pore filters. The filtrate after each filtration and the residue on the 104 μm mesh filter was searched under the dissection microscope as described above.

3.2.2.4. Enzymatic and micro-dissection isolation

Cortical slices were incubated in dissection medium containing either 0 or 3 mg/ml collagenase at 37 ^{o}C for 30 minutes. The slices were examined under the dissection microscope and using 25 G needles connected to syringe barrels, any observed preantral follicles were isolated taking care not to rupture the basement membrane.

Follicles collected were fixed overnight in Bouins solution and stained with haemotoxylin and eosin (see chapter 2). The sections were examined under the microscope (Nikon Microphot, Nikon, Japan). Follicle and oocyte diameters were measured and the number of granulosa cell layers recorded. In addition, follicles were classed as having either no antrum, an early antrum or an antrum present.

3.2.2.5. Manual isolation of granulosa oocyte complexes

Cortical sections were obtained as described above. Preantral follicles were carefully burst open using 25 G needles and the granulosa oocyte complexes released were collected using a pipette.

3.2.3. Maintenance of morphology during culture

Follicles collected by the above methods were placed in 24 well tissue culture plates (Sterilin, Hounslow, UK.) with a surface coating of collagen (for collagen mix preparation see chapter 2). 1 ml of culture medium (for preparation see chapter 2) was used to wash the collagen before fresh medium was added. Culture wells were incubated at 39 °C, 5 % CO_2 in a humidified incubator for at least one hour before the follicles were added. Between 4 - 6 follicles were added to each culture well and cultured at 39 °C, 5 % CO_2 in a humidified incubator for 7 days. Half the medium was changed on days 1, 3 and 5 of culture. Follicle measurements were made using a calibrated eyepiece graticule fitted to an inverted microscope (Nikon Daiphot, Nikon) on days 0, 1, 3, 5 and 7 of culture. Follicles becoming obviously degenerate were excluded from the results.

3.2.4. Analysis of results

Results were analysed initially by a one way analysis of variance using the statistical package Minitab, version Plus/SE. Significant differences between results were determined by a Student's t-test.

3.3. RESULTS

3.3.1. Localisation of preantral follicles within the ovary

Examination of transverse sections of bovine ovaries revealed that most preantral follicles are found just under the surface of the cortex (figure 3.1). No follicles were found within the outer region of the cortex (figure 3.1). Examination of cortical slice sections showed that follicles were present on the inner surface of the cortex, making them readily available for isolation by micro-dissection (figure 3.1).

3.3.2.Follicle Isolation

3.3.2.1. Enzymatic digestion

With this method, incubation of the cortical slices with at least 3 mg/ml of collagenase was required before any follicles were found in the filtrate. The follicles collected were few in number (maximum of 10 retrieved from 1 ovary) and smaller (typically less than 110 μm) than required.

3.3.2.2. Enzymatic digestion and homogenisation and mechanical isolation

Neither of these methods were successful in isolating large numbers of follicles. This was because the residue, when searched, contained large amounts of stromal tissue and follicle fragments making it difficult to find any intact follicles under the microscope. Follicles which were found were between 47 μm and 117 μm in diameter and therefore smaller than required for this study.

In addition, follicles obtained by this and the previous method were found difficult to handle because they readily stuck to plastics used in the pipette tips and petri dishes (both tissue culture and non tissue culture treated dishes).

3.3.2.3. Manual isolation of granulosa oocyte complexes

Bursting of follicle walls and collection of the intact granulosa cell-oocyte complex (GOC) proved difficult and frequently resulted in a naked oocyte being released. Intact GOC retrieved were easily stripped of the granulosa cells during transfer to fresh medium using pipettes. Despite this, a number of follicles were obtained for preliminary culture studies.

3.3.2.4. Enzymatic and manual isolation

This method allowed up to 40 follicles to be isolated from a single ovary. A large amount of variation was observed between ovaries in terms of the number of follicles isolated which did not appear to be related to the general gross morphology of the ovary. The follicles isolated (figure 3.2) were approximately 60 μm - 380 μm in diameter (figure 3.3) containing oocytes ranging from 20 μm to 90 μm in diameter (figure 3.3) and had a surrounding layer of stromal tissue. Preincubation with collagenase did not have a detectable effect on the ease of dissection and it was observed that follicles in this group ruptured more easily during dissection than those not incubated with collagenase. For this reason, treatment with collagenase was not used for collection of follicles for culture. Dissection was continued for up to 3 hours after the initial isolation of cortical slices, after which time, dissection of intact follicles without bursting became increasingly difficult even in the absence of collagenase.

Histological analysis of sections from micro-dissected follicles showed a strong positive correlation between follicle and oocyte diameter (figure 3.3). Classification of follicles on the basis of antrum class (figures 3.4, 3.5) showed that antrum formation occurred between mean follicle diameters 171.6 μm - 242.2 μm. Follicles without an antrum were significantly smaller than those with either an early antrum or an antrum (figure 3.4, $p<0.01$). Antral cavities were never observed in follicles less than 139.7 μm in diameter and were always observed in follicles with diameters greater than 232.6 μm. Oocytes in preantral follicles were slightly smaller

than those of antral follicles (figure 3.4, $p<0.1$). No significant differences were detected in the number of granulosa cell layers between the preantral, early antral and antral follicles (figure 3.4).

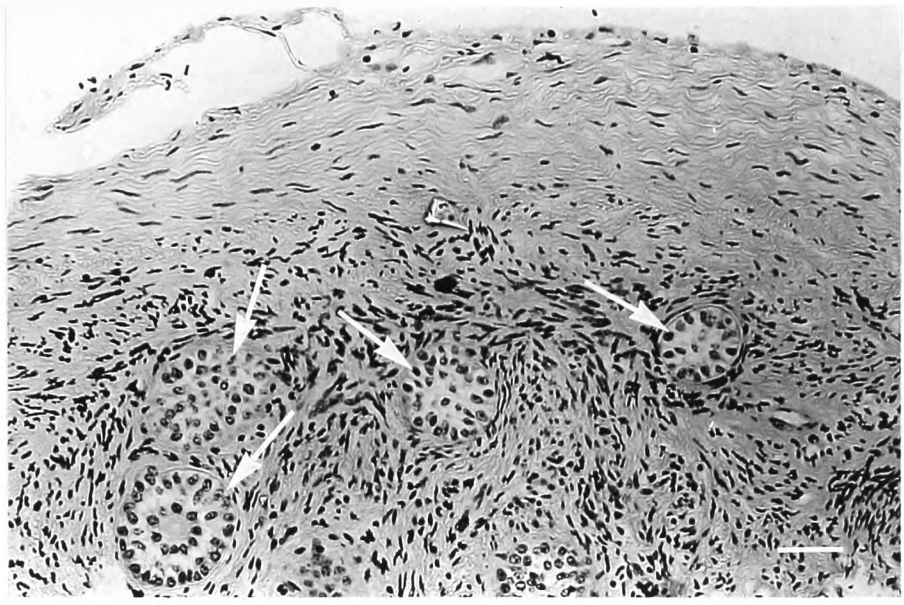
3.3.3. Maintenance of morphology during culture

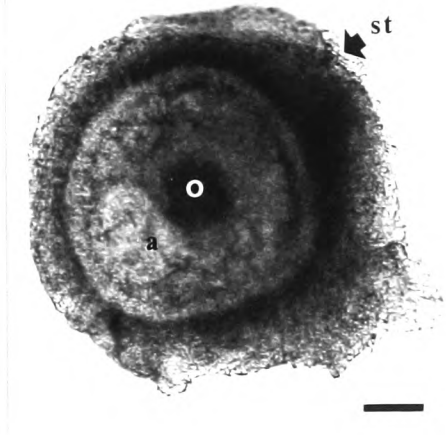
GOC or intact follicles collected by manual dissection were cultured for up to 7 days. The oocytes of the GOC were surrounded by granulosa cells up until day 3 of culture after which all oocytes became denuded and granulosa cells had plated down to form a monolayer (figure 3.6). By day 5 of culture oocytes were significantly smaller than at the start of culture (table 3.1, $p<0.05$), indicating degeneration of the oocytes when not surrounded by granulosa cells.

Intact micro-dissected follicles maintained their morphology over the duration of culture (figure 3.6) and a significant increase in follicle diameter during the culture period was observed (figure 3.7, $p<0.05$). A small proportion of follicles became degenerate during culture rising from 3 % on day 3 to 13 % on day 7.

Figure 3.1. Histological section, stained with haemotoxylin and eosin, of the cortex of the bovine ovary. Most preantral follicles (arrows) were located in the inner cortex. Follicles were never seen in the outer cortical tissue. Bar represent 50μm.

Figure 3.2. Bovine follicle obtained by micro-dissection. The basement membrane is intact and the oocyte (o) is visible. Stromal or theca tissue (st) is adherent on the outer surface of the follicle and a small antral cavity is visible (a). Bar represent 50μm.

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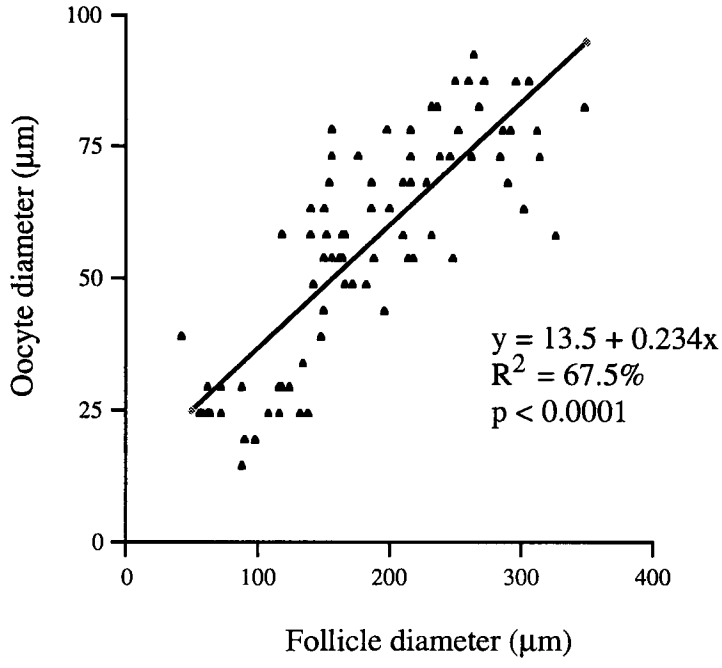


Figure 3.3. Scatter plot showing the relationship between oocyte and follicle diameter of freshly microdissected follicles (n=80 follicles). Oocyte and follicle diameters were measured on the nucleolus containing section. Regression analysis showed a positive correlation between oocyte diameter and follicle diameter.

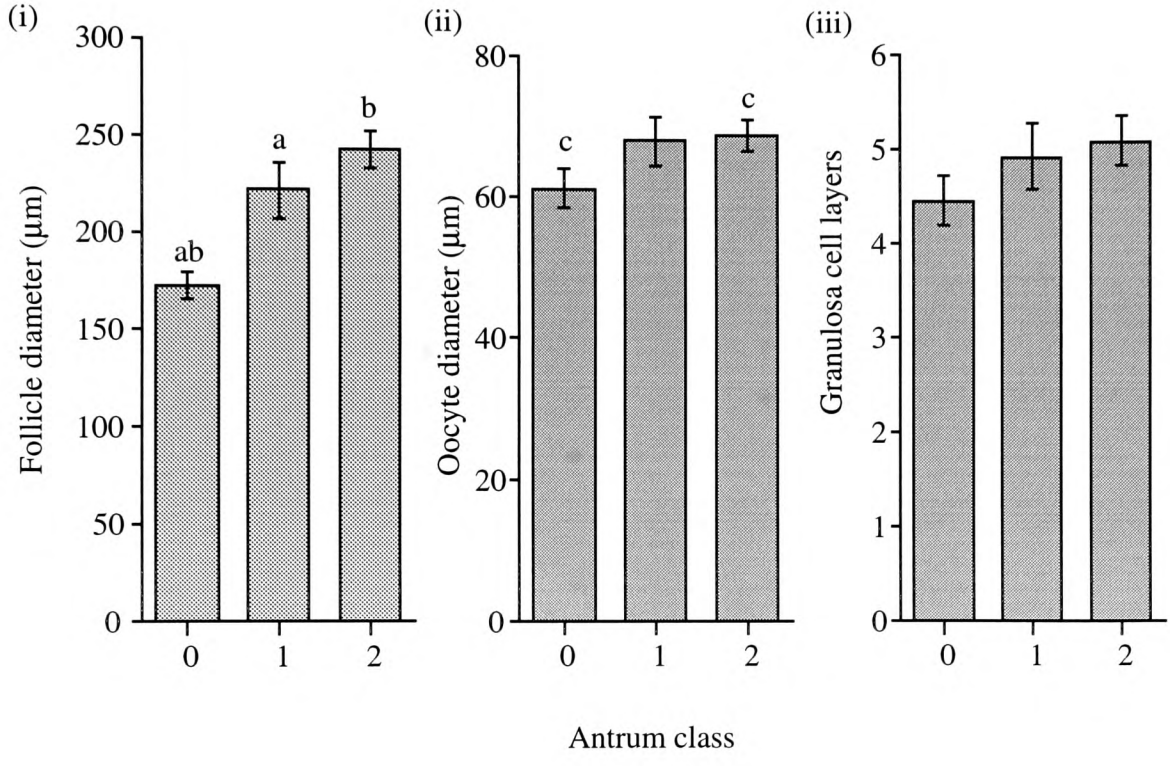


Figure 3.4. Mean (i) follicle diameter, (ii) oocyte diameter and (iii) number of granulosa cell layers in histological sections of microdissected bovine preantral follicles grouped by antrum class (0 = no antrum (n = 19 follicles), 1 = early antrum (n = 12 follicles), 2 = antrum present (n =14 follicles) in the oocyte nucleolus containing section). Significantly different results between classes are shown by the letters a and b ($p<0.01$), possible differences are shown by the letter c ($p<0.1$), as determined by a Student's t-test. Results are means ± s.e.m.

Figure 3.5. Histological sections, stained with haemotoxylin and eosin, of follicles classified by the presence or absence of an antrum. a. Class 0 (no antrum), b. class 1 (early antrum), c. class 2 (antrum). Bar represents 50 μm.

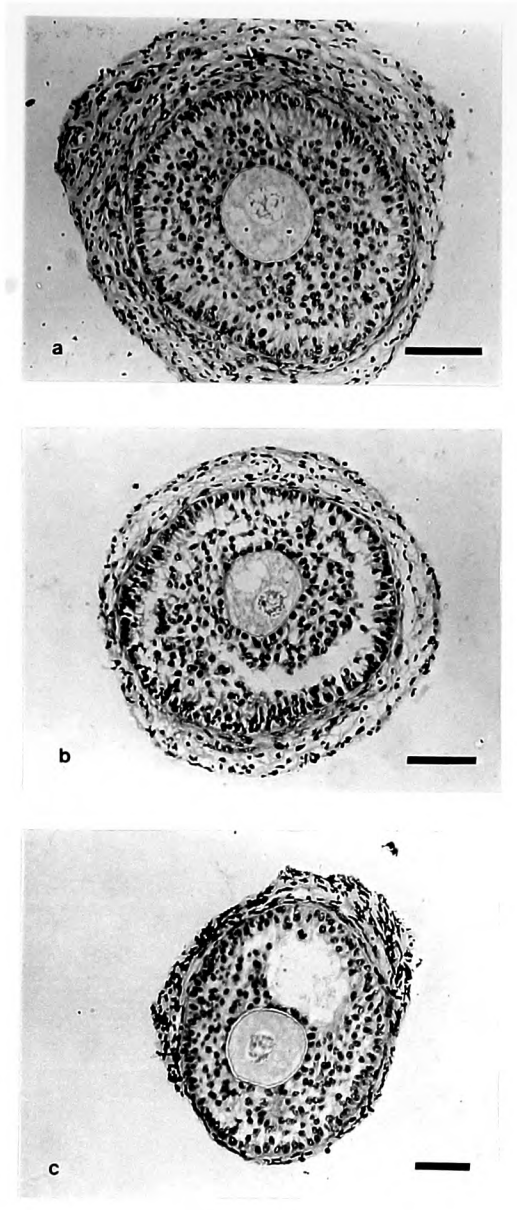
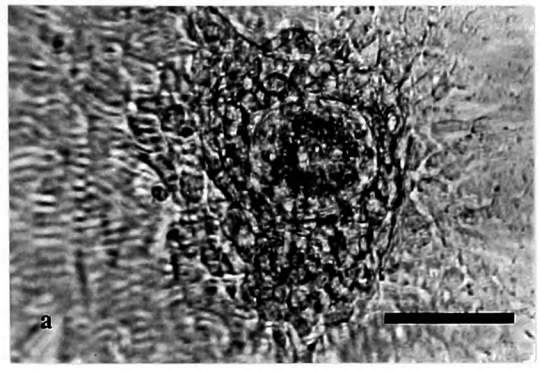
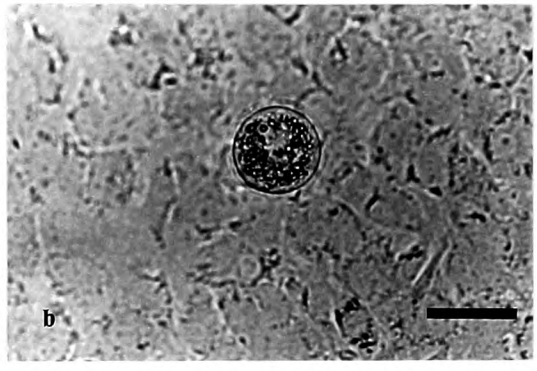
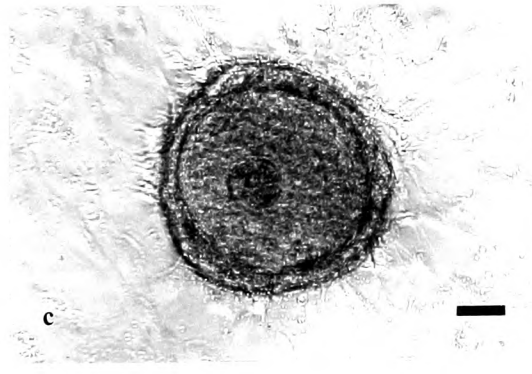

a
b
c

Figure 3.6. Photomicrograph of a granulosa cell-oocyte complex (GOC) following culture for a. 1 day and b. 4 days. After 1 day the granulosa cells attach to the substratum and by day 4, a monolayer has formed and the oocyte has become denuded. In contrast, following culture of an intact, micro-dissected preantral follicle for 7 days (c.), follicle morphology is maintained. Bars represent 50μm.


a


b


c

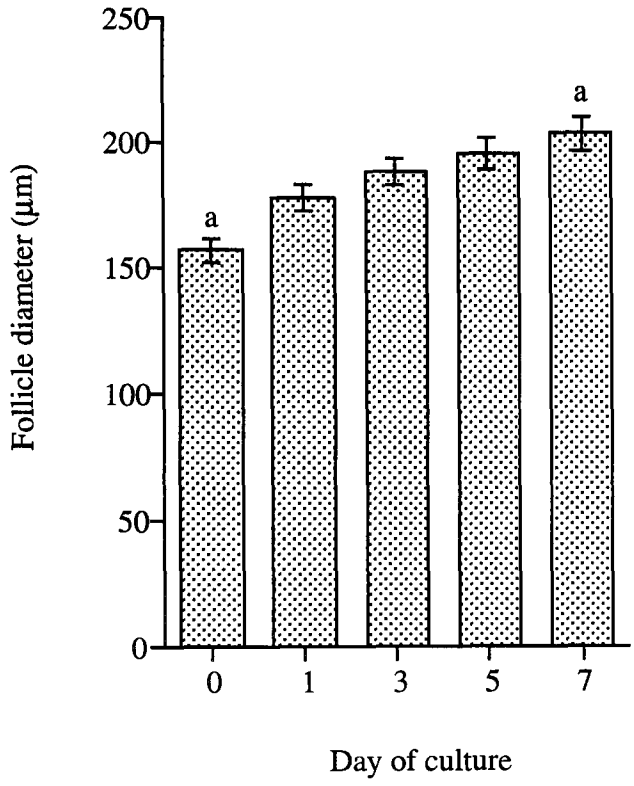


Figure 3.7. Histogram showing the growth of preantral follicles (n = 60), isolated by microdissection of cortical slices, during culture for 7 days.

[a]Follicles on day 7 of culture were significantly larger ($p<0.05$) than at the beginning of culture (day 0) as determined by a Student's t-test. Results are mean ± s.e.m.

Table 3.1. Mean oocyte diameter during the culture of granulosa cell-oocyte complexes (GOC) (n = 14) for 5 days.

Day of culture	Oocyte diameter (μm)[a]	s.e.m.
0	61.2	2.1
1	66.4	3.0
3	56.1	3.2
5	51.0	2.5

[a] Oocytes on day 5 were significantly smaller than oocytes on day 0 ($p<0.05$) as determined by a Student's t-test.

3.4. DISCUSSION

In this study, we have evaluated a number of possible methods for the isolation of large preantral follicles. We have demonstrated that micro-dissection of cortical slices, without the use of enzymatic digestion, yields morphologically normal intact large preantral follicles. Preliminary culture studies have shown that the follicles maintain their morphology over 7 days.

Analysis of sections taken from bovine ovaries have shown that most preantral follicles are found within the ovarian cortex, therefore slices of ovarian cortex were removed from the ovary to evaluate a number of isolation methods.

For the first time, this study has demonstrated successful micro-dissection of cortical slices to isolate bovine large preantral follicles. Through avoiding damage by vigorous mechanical or enzymatic treatments, micro-dissection of intact follicles provided a more physiologically relevant model for examining follicular development. Although micro-dissection has been used successfully to isolate murine large preantral follicles (Nayudu and Osborn, 1992, Boland et al., 1993), adaptation of this technique to domestic mammals has been hindered by a larger, more fibrous ovary, making dissection difficult. To overcome these problems, this study used cortical slices, identified as a rich source of preantral follicles, thin enough for individual follicles to be seen under the dissection microscope.

Histological study of micro-dissected intact follicles allowed detailed characterisation of their morphology. The follicles isolated by this method had an intact basement membrane, several surrounding stromal or theca layers and a visible oocyte. Antral cavities could be seen in some follicles indicating that isolation did not disrupt the intra-follicular architecture. The follicle diameters at which antrum formation occurred corresponds to previous studies of cattle ovaries (Lussier et al., 1987, Monniaux et al., 1984). In addition, classification of antrum classes showed that, as the antrum developed in the follicles studied, follicle and oocyte diameters

increased in diameter but not the number of granulosa cell layers. This indicates that during antrum formation, increases in follicular diameter may be attributed more to increases in oocyte and antrum size than increases in the number of granulosa cell layers, although further work is required to substantiate this.

Micro-dissected bovine preantral follicles maintained their morphology and increased in follicle diameter over the 7 day culture period with few follicles becoming degenerate. Micro-dissected murine preantral follicles have undergone growth in vitro to produce developmentally competent oocytes (Boland et al., 1993, Spears et al., 1994) confirming the suitability of the isolation method to provide follicles for examining murine follicular development in vitro. From the results obtained in this preliminary experiment, it would appear that micro-dissected preantral follicles may also be an appropriate starting material for the in vitro growth of bovine preantral follicles.

This study found that mechanical disruption of ovarian cortical slices fails to isolate intact large preantral follicles. Fragments of follicles were found using these methods and it is likely that the mechanical isolation methods cause rupture of larger follicles. Rapid mechanical methods have been devised which allow large numbers of bovine small preantral follicles (<100 μm in diameter) to be isolated from a single ovary (Jewgenow and Pitra, 1991, Nuttinck et al., 1993, Figueiredo et al., 1993). In agreement with our results, these authors found that these methods were not successful in isolating large preantral follicles.

Mechanical methods for small preantral follicle isolation have been successful when applied to foetal bovine ovaries, with ovaries from mature animals yielding far fewer follicles (Nicosia et al., 1975, Figueiredo et al., 1993). Ovaries from mature cattle are larger and contain fewer follicles than those of foetal ovaries (Figueiredo et al., 1993). After homogenisation of ovarian tissue from mature cattle, separation of the small number of follicles from the large amount of interstitial tissue

was difficult. A further advantage of using foetal ovaries from domestic animals is the possibility of increasing rates of genetic improvement by fertilisation of in vitro grown oocytes allowing reduced generation intervals (Betteridge et al., 1989). Foetal ovaries were not used in this study because of irregular availability.

In this study, collagenase pretreatment of cortical slices before micro-dissection did not aid follicle isolation and often resulted in follicle rupture during dissection. In contrast, collagenase digestion of bovine ovaries has increased the recovery of small preantral follicles (Figueiredo et al., 1993). Isolation of small follicles using collagenase is easier because of the lack of intimate association with theca cells which prevents rapid isolation (rabbit: Nicosia et al., 1975), thereby shorter digestion times are required resulting in reduced damage to the basement membrane. In our study, it was likely that the duration and concentration of collagenase as well as the difficulty in neutralising its action resulted in excessive digestion of the basement membrane of large preantral follicles during isolation. However, even without the use of collagenase, micro-dissection of follicles without basement membrane rupture became increasingly difficult with length of dissection presumably due to enzymes released by the rupture of cells.

Apart from the problems of basement membrane rupture caused by collagenase isolation, collagenase has recently been reported to have a detrimental effect on the bovine oocyte (Nuttinck et al., 1996, Wandji et al., 1996a). It is unclear whether this is as direct effect of collagenase on the bovine oocyte or an indirect effect caused by the disruption of granulosa cell - oocyte communication which is necessary for oocyte development (Eppig, 1979a). Collagenase isolation has been successfully used on murine preantral follicles without disruption of granulosa cell oocyte communication (Eppig and Downs, 1987, Heller et al., 1981). These follicles have then been grown in vitro to produce developmentally competent oocytes (Eppig and

Schroeder, 1989). It is apparent therefore that species differences in the sensitivity of the oocyte to collagenase exist.

Isolation and culture of murine follicles as GOC is the most successful method for the production of large numbers of developmentally competent in vitro grown oocytes (Eppig and Schroeder, 1989). An enzymatic isolation procedure (Eppig and Downs, 1987) is used to produce GOC, which are essentially a follicle devoid of a basement membrane and theca tissue (for review see: Eppig et al., 1996). In this study a manual isolation procedure was used to collect bovine GOC which combined the advantages over the enzymatic isolation system of (i) not using collagenase and (ii) allowed selection on the basis of follicle size and morphology. Retrieval rates using this method were slightly lower than the micro-dissection technique due to the ease of the oocyte becoming denuded during removal from the follicle. However, sufficient numbers were collected to conduct trial cultures.

Bovine GOC when cultured for 5 days in conditions similar to those used for the successful growth of murine GOC (Eppig and Schroeder, 1989), resulted in the granulosa cells plating down onto the collagen substrate to form a monolayer after 3 days in culture. This caused the oocyte to become denuded and decrease in size such that it was significantly smaller than at the start of culture. Communication of the oocyte with the granulosa cells is known to be important for normal oocyte development (Eppig, 1979a, Heller et al, 1981, Buccione et al., 1994). It is likely that the oocyte becoming denuded is associated with its degeneration and failure to grow during culture. Encapsulation of bovine GOC in a collagen matrix, as demonstrated successfully for murine (Torrance et al., 1989, Carroll et al., 1991a) or porcine (Hirao et al., 1994) preantral follicles may be a more suitable method of culture.

In summary, a number of techniques for the isolation of bovine large preantral follicles were examined. Micro-dissection of cortical slices, without the use of enzymatic digestion, yielded morphologically normal intact large preantral follicles

which were a suitable starting material for culture. Preliminary studies showed that the follicles maintained their morphology during culture for 7 days. It is hoped to use this method of isolation and culture as a model for studies of bovine follicular development.

CHAPTER 4. DEVELOPMENT OF A CULTURE SYSTEM FOR BOVINE PREANTRAL FOLLICLES

4.1. INTRODUCTION

Derivation of suitable culture conditions required for in vitro growth of bovine ovarian follicles will require a number of factors to be examined. Type and availability of nutrients and hormones and the nature of the substrate support need to be considered. This series of experiments will examine the effects of these factors on the in vitro growth of bovine preantral follicles with a view to defining suitable culture conditions for subsequent studies.

Whether interfollicular effects are important during bovine preantral follicle development is not yet known and must be considered when deciding to culture follicles singly or in groups. Interactions between large antral follicles are important for selection of the bovine preovulatory follicle (for reviews see Ireland, 1987, Fortune, 1994). Studies using cultures of isolated murine preantral follicles have indicated that similar mechanisms may affect the development of smaller follicles (Nayudu and Osborn, 1992, Spears et al., 1994) and could be play a role in the regulation of spacing between follicles (Nayudu and Osborn, 1992). The effect may be mediated by a complex interaction between secreted factors and direct follicle-follicle contact (Nayudu and Osborn, 1992, Spears et al., 1994). The presence of follicle interactions may complicate the analysis of factors influencing follicular development.

A wide range of medium volumes have been used to culture preantral follicles (e.g. mouse: 20 µl/follicle (Boland et al.,1993), pig: 600 µl/follicle (Hirao et al., (1994)) The effect of different volumes of culture medium on isolated bovine

preantral follicle growth in vitro have not yet been analysed. The volume of follicle culture medium must be large enough to provide adequate nutritional support and allow the accumulation of waste products. In addition, a build up of follicle produced factors (e.g. growth factors, steroids) may also influence follicular development as has been shown in murine preantral follicle cultures where reduction of medium volume improved preantral follicle growth (Qvist et al., 1991).

The optimum combination of medium volume and number of medium changes for a given number of bovine preantral follicles is unknown. Nutritional factors in the follicle culture medium will be depleted either by utilisation or deterioration and it will be necessary to replenish the medium if prolonged culture is to be achieved. Beneficial effects of medium changes during murine preantral follicle culture showed increased rates of follicular growth if the medium was changed more frequently (Nayudu and Osborn, 1992). The requirement for nutritional factors will be greater as the number of follicles present is increased and either more medium changes or a larger culture medium volume will be necessary.

A number of nutritional factors are necessary for normal follicular development. Serum is used as a general nutrient supplement to culture medium for a large number of cell types. In vitro growth of follicles can be achieved in the absence of serum (Eppig et al., 1992, Hulshof et al., 1995), however, rates of follicle growth, oocyte developmental competence and morphological quality are reduced (Eppig et al., 1992, Hulshof et al., 1995, Wandji et al., 1996a). Culture with serum has been used successfully in a number of murine follicle culture systems (Nayudu and Osborn, 1992, Boland et al., 1993, Eppig and Schroeder, 1989, Torrance et al., 1989). The components of serum are numerous, and the factors which affect follicle growth may be a combination of nutrients, growth factors and hormones. However, the presence of serum complicates analysis of factors effecting follicular development in vitro. Nevertheless, the present serum-free follicle culture systems are less effective at maintaining follicle development in vitro (Eppig et al., 1992, Hulshof et al., 1995).

Insulin stimulates the proliferation of cultured granulosa cells (Gong et al., 1993). However, culture of intact follicles either isolated (mouse: Spears et al., 1994b) or as cortical slices (cow: Peluso and Hirschell, 1988) have failed to show a stimulatory effect on proliferation. The reason for this is unclear, but may involve an interaction with the regulation of granulosa cell proliferation by the oocyte (Vanderhyden et al., 1992). Nevertheless, insulin has been shown to be important for maintenance of follicle viability (Roy and Greenwald, 1989).

Intercommunication between granulosa cells and the oocyte is important for normal follicular development (Eppig, 1991). The purine, hypoxanthine, has been shown to preserve the gap junctional communication between the oocyte and granulosa cells necessary for intercommunication between these 2 cell types (Eppig and Downs, 1987, Heller et al., 1981). Some culture media (e.g. M199) contain a limited amount of hypoxanthine. Further supplementation of medium with hypoxanthine may have a beneficial effect on follicle development.

The basement membrane separates the avascular follicle from the vascularised interstitial tissue. As the follicle grows from the primordial to preovulatory stages, it must increase hugely in size. Cells on both sides of the basement membrane are thought to be involved in its deposition (Luck et al., 1995) which involves the synthesis of a number of components including collagen type IV, lamanins and fibronectin (Olsen and Ninomiya, 1993, Hynes, 1993, Engvall, 1993).

The substrate on which the follicle grows in vitro must maintain the integrity of the basement membrane either directly or indirectly through the maintenance of granulosa or theca cells. In vitro growth of murine follicles on substrates allowing basement membrane rupture result in the follicle cells spreading away from the oocyte and disruption of the follicle architecture (Cain et al, 1995, Daniel et al., 1989, Maresh et al., 1990). Collagen (type I) has been used as a substrate for the growth of follicles as it is the major component of the ovarian extracellular matrix (mouse: Eppig and Telfer, 1993, cow: Figuiredo et al., 1994, pig:

Hirao et al., 1994, Donelley and Telfer, 1994). Intact follicles grown on collagen attach, but do not spread significantly, maintaining the organisation of the granulosa cells around the oocyte. Despite the basement membrane being of critical importance to follicular development very little is known about its deposition and turnover as the follicle develops. In vitro growth of intact follicles provides an excellent opportunity to examine this process.

In this study, culture conditions suitable for examining bovine preantral follicle growth will be determined by: (i) defining the interaction between follicle numbers , volume of medium and the number of changes of medium (ii) assessing the effect of nutrient supply by culture with serum, insulin and hypoxanthine in the medium (iii) determining whether stromal tissue, the substrate for in vivo follicular growth, or collagen, a major component of the extracellular matrix in vivo, is more suitable substrate

4.2. METHODS

4.2.1. Follicle collection

Large preantral follicles were isolated by micro-dissection of ovarian cortical slices as described previously (chapter 2).

4.2.2. Effect of volume of culture medium, number of medium changes and the number of follicles per culture well on preantral follicle growth.

Four well tissue culture plates coated with collagen were prepared (chapter 2). Groups of 1 or 4 follicles were added to either 0.25ml or 1ml of culture medium (chapter 2). Follicles cultured as groups of 4 were evenly spaced on the collagen gel substrate. Follicles were cultured for 7 days, during which time follicle and oocyte diameters were measured on days 0, 1, 3, 5 and 7 using an inverted microscope (Nikon Diaphot, Nikon, Japan) fitted with a crossed eyepiece graticule (Graticules Ltd., Tonbridge, UK). Half the culture medium was changed 0, 3 or 6 times during the culture period. If 3 changes were to be made, this was done on days 1, 3 and 5 whilst 6 changes necessitated changes on all days of culture.

4.2.2.1. Experimental design

Treatments were assigned to 1 of six experimental groups (table 4.1). The experiment was based on a 3 x 2 x 2 factorial design. 2 experimental groups in duplicate were set up at a time, with any extra follicles cultured under the appropriate single follicle conditions.

Differences in the means between treatment groups were detected using a Student's t-test on Minitab. A factorial analysis of variance using the REML (restricted maximum likelihood) model (which allowed analysis between treatments with unequal numbers of observations) was also carried out on Genstat (version 5,

release 3.2) which analysed the effects of medium volume, number of medium changes and the number of follicles per well and their interactions.

4.2.3. The effect of serum on preantral follicle growth

Four well tissue culture plates coated with collagen were prepared (chapter 2). Groups of 4 follicles were added to culture medium (chapter 2) containing either 10% heat inactivated foetal calf serum (Globepharm, Surrey, UK) or 3mg/ml BSA (bovine fraction V, Sigma, Dorset, UK)). Culture was continued for 7 days. Follicle and oocyte diameters were measured using an inverted microscope on days 0,1,3, 5 and 7 of culture. Half the medium was replaced on days 1, 3 and 5 of culture. On day 7, follicles were fixed in Bouins solution and processed for histology (chapter 2). Haemotoxylin and eosin stained sections were examined under the light microscope and the number of pyknotic cells and presence of an antrum recorded. Follicle and oocyte morphology were scored where 0 = degenerate, 1 = possibly degenerate and 2 = normal. Normal oocytes were spherical in shape, had an intact germinal vesicle and close contact with granulosa cells. Normal follicles were regularly shaped, with few pycnotic granulosa cells, an intact basement membrane and a healthy oocyte.

4.2.4. The effect of insulin on preantral follicle growth

Four well tissue culture plates coated with collagen were prepared (chapter 2). Groups of 4 follicles were added to culture medium (chapter 2) containing either 0 or 50μg/ml insulin (Sigma). Culture was continued for 5 days, during which follicles were measured on days 0, 1, 3 and 5 and half the medium changed on days 1 and 3.

4.2.5. The effect of hypoxanthine on preantral follicle growth

Four well tissue culture plates coated with collagen were prepared (chapter 2). Groups of 4 follicles were added to culture medium (chapter 2). Hypoxanthine (Sigma) was added to the culture medium at a concentration of 2mM, culture of

follicles in medium without additional hypoxanthine was used as a control. Culture was continued for 5 days, during which follicles were measured and medium changed as described above.

4.2.6. Comparison of stromal cells or collagen as a substrate for follicle growth

4.2.6.1.Preparation of stromal cell substrate

4.2.6.1.1. Isolation of stromal cells

Pieces of stromal tissue were micro-dissected from cortical slices using 25G needles attached to syringe barrels and incubated with 1 ml of trypsin/EGTA solution (100ml/l 10X, 2.5% trypsin (Life Technologies, Paisley, UK), 100mM sodium chloride, 0.7mM di-sodium phosphate, 1.6mM potassium hydrogen phosphate, 4.5 mM potassium chloride, 5 mM D-glucose, 22mM tris, 0.9ml/l pheno red (all BDH, Poole, Dorset, UK), 0.4g/l EGTA, 0.1g/l P.V.A. (both Sigma) dissolved in distilled water, and pH adjusted to pH7.6 by the addition of 1 M HCl or 1 M NaOH) for 2 hours at 39°C. Disaggregation of cells was aided by repeated pipetting. 4ml of tissue culture medium (Glasgow MEM, 1.12mM sodium pyruvate, 5.6ml/l non-essential amino acids (all Life Technologies, Paisley, UK), 5% foetal calf serum, 5% new born calf serum (Globepharm, Surrey, UK)) was added to inactivate the trypsin and the cell suspension was then centrifuged at 1000g for 5 minutes. The overlying medium was drawn off using a Pasteur pipette connected to a suction pump and the pellet of cells resuspended in 10ml of tissue culture medium.

4.2.6.1.2. Preparation of cell stocks

The cell suspension was added to an 80ml tissue culture flask (Nunc, Roskilde, Denmark) and cultured for 3 days at 39°C, 5% CO_2. Cells were passaged on day 3 after removing the culture medium and rinsing the cells with 5ml of PBS to

remove the serum. Cells were passaged by adding 1.5ml of trypsin/EGTA solution to the flask and incubating for 3 minutes, or until the cells begin to become rounded and detach from the surface of the flask. The flask was agitated to loosen the cells and 4 ml of tissue culture medium added to neutralise the trypsin. The cells were resuspended by repeated pipetting using a Pasteur pipette until a single cell suspension was obtained. The cells were then spun at 1000g, the overlying medium removed, and the cells resuspended in 5ml fresh tissue culture medium. 0.5ml of the cell suspension was added to an 80ml tissue culture flask and again cultured to confluence. These cells were also passaged and replated in a 175ml flask (Nunc).

4.2.6.1.3. Freezing and storage of cell stocks

After the 3rd passage, the cells were resuspended in 2.5ml of culture medium and 2.5ml of freezing mix (culture medium supplemeted with 20% DMSO (Sigma)) and 0.5ml aliquots were added to precooled (-80^{o}C) freezing vials. Aliquots were initially stored at (-80^{o}C) overnight and then liquid nitrogen until required.

4.2.6.1.4. Resuscitation of cells

When required, cells were resuscitated by warming the vials rapidly to 39^{o}C in a water bath, resuspended in 1ml of tissue culture medium and added to 20ml of tissue culture medium in an 80ml flask and grown to confluence after which a cell suspension was prepared as described above.

4.2.6.1.5. Preparation of monolayers for follicle culture

0.5ml of the cell suspension was added to 5ml of fresh tissue culture medium. 0.5ml of this (or less if the cell suspension is dense) was added to the culture wells (4 well plates (Nunclon, Roskilde, Denmark) 1 day before the follicles are added. Immediately prior to culture, the tissue culture medium was replaced with 1ml of culture medium for follicle growth (chapter 2)

4.2.6.2. Preparation of collagen substrate

4 well culture plates (Nunclon, Roskilde, Denmark) were coated with a collagen mix prepared from rats tails (chapter 2). 1ml of culture medium was added to each well.

4.2.6.3. Follicle culture

4 follicles were added to each well and culture was continued for 5 days. Follicles were measured and medium changed as described above.

4.2.7. Experimental design and statistical analysis (serum, insulin, hypoxanthine and substrate experiments)

Within each experiment, on any one day, follicles were isolated from 1 - 2 ovaries and randomly assigned to each treatment group within an experiment. Results were analysed on an individual follicle basis using a Student's t-test with the statistical analysis package Minitab (version Plus/SE).

Experimental group	Number of medium changes during 7 days of culture	Volume of medium/well (ml)	Number of follicles/well
A	0	0.25	1
		1	4
B	3	0.25	1
		1	4
C	6	0.25	1
		1	4
D	0	1	1
		0.25	4
E	3	1	1
		0.25	4
F	6	1	1
		0.25	4

Table 4.1. Experimental groups used for volume of medium/well, number of medium changes and number of follicles/well experiment. 2 experimental groups were set up each day in duplicate. All possible combinations of experimental groups were examined.

4.3. RESULTS

4.3.1. Effect of culture medium volume, number of medium changes and number of follicles per culture well on preantral follicle growth.

Figures 4.1 and 4.2 and tables 4.2-4.3 show increases in follicle and oocyte diameters after 7 days of culture. The same follicle or oocyte measurements are presented more than once, although organised differently, so that the relevant comparisons between the number of follicles per well, number of medium changes or volume of culture medium can be clearly shown. Clear and consistent effects of culture conditions became apparent between 5 - 7 days of culture, therefore only the results from day 7 are presented here.

4.3.1.1. Growth of follicles and oocytes

Under all treatments, mean follicle diameters increased in size after 7 days of culture (figure 4.1). After 7 days of culture mean oocyte diameter was at least maintained at sizes similar to those at the start of culture (table 4.2). These results may be slightly lower than the true oocyte diameter as the oocyte was often difficult to see on day 7. In those cases, the corresponding oocyte diameter from day 5 was used for calculating the mean oocyte diameter.

Follicle and oocyte diameters were not used for analysis if: (i) oocytes were considered degenerate when oocyte diameters could not be measured on 2 consecutive measurement days (this happened to 32 out of 321 follicles (10%)), (ii) the oocyte was extruded from the follicle (this happened to 24 out of 321 follicles (7.5%)), (iii) the follicle was obviously degenerate (this happened to 23 out of 321 follicles (7.2%)) and (iv) there was a bacterial or fungal infection of the culture (this happened to 1 follicle out of 321 (0.3%)). Reasons (i), (ii) and (iii) happened in all experimental groups and no obvious trend in the distribution was noticed.

4.3.1.2. The effect of the number of follicles/well

Culture with 1 or 4 follicles per well was examined under different medium changing routines and volumes of medium (table 4.3). No significant differences on the increase in follicle or oocyte diameters after 7 days of culture were detected ($p>0.05$, table 4.3) between follicles grown singly or as groups of 4 when comparing cultures under the same volume of medium per follicle and medium changing routine (table 4.3).

4.3.1.3. The effect of number of medium changes

Half the culture medium was changed 0, 3 or 6 times during culture. No significant differences in the increase in follicle or oocyte diameter after 7 days of culture were detected between changing the medium 3 or 6 times ($p>0.05$, figure 4.1, table 4.2). However, if the medium was not changed, follicles but not oocytes increased in size significantly more than the 3 or 6 medium change groups only if the medium volume was 0.25 ml for 1 follicle/well or 1 ml for 4 follicles/well ($p<0.05$, figure 4.1).

4.3.1.4. The effect of volume of culture medium

The volume of medium in the culture well only had a significant effect on the increase in follicle diameter on day 7 and when 0 changes of medium were made ($p<0.05$, figure 4.2). This effect differed depending on the number of follicles in the well. With 1 follicle/well, the increase in follicle diameter was significantly greater in 0.25ml compared to 1ml of culture medium ($p<0.05$, figure 4.2.). In contrast, with 4 follicles/well the increase in follicle diameter was significantly greater in 1ml compared to 0.25ml of culture medium ($p<0.05$, figure 4.2.) (see also section 4.3.1.5.). No effect of the volume of culture medium on the increase in oocyte diameter during culture was observed ($p>0.05$, table 4.2).

4.3.1.5. Interaction between number of follicles/well, medium changes and medium volume

4.3.1.5.1. Follicle diameter

The 3 way interaction between medium volume, number of changes and follicle number on the increase in follicle diameter by day 7 was not found to be significant overall as determined by a factorial analysis of variance. Two large, but not significant negative effects of 3 or 6 medium changes were found when there were 4 follicles per well in 1ml of medium.

The only significant 2 way interaction was between the number of follicles per well and the volume of medium. The combination of these 2 factors means that when there were no medium changes, it was better to have 1ml of medium for 4 follicles per well and 0.25ml of medium for 1 follicle per well. Three or 6 medium changes overcame any follicle number/medium volume interaction.

4.3.1.5.2. Oocyte diameter

Factorial analysis of variance did not detect any significant interactions between number of follicles, volume of medium and number of changes on the increase in oocyte diameter by day 7.

4.3.2. The effect of serum on preantral follicle growth

Culture of preantral follicles in serum resulted in significantly larger follicles than those cultured without serum by day 3 of culture ($p<0.05$) (figure 4.3). This difference in size was maintained until the end of culture on day 7. The presence or absence of serum did not significantly effect the growth of oocytes ($p>0.05$) (figure 4.3) although it was noted that the variation in oocyte diameter was greater when serum was not included in the culture medium (figure 4.3).

Analysis of histological sections showed that the effect of serum on follicle growth was likely to be the result of an increase in granulosa cell proliferation as the

number of granulosa cell layers was greater ($p<0.01$) (figure 4.4). Serum did not significantly alter the number of pyknotic cells, follicle quality, oocyte quality or proportion of follicles with an antrum ($p>0.05$) (table 4.4).

4.3.3. The effect of insulin on preantral follicle growth

The addition of insulin to the culture medium stimulated follicle growth by day 3 of culture at levels approaching significance ($p<0.1$) (figure 4.5) and both follicle and oocyte growth by day 5 of culture (figure 4.5).

4.3.4. Effect of additional hypoxanthine on preantral follicle growth

Culture in the presence of hypoxanthine did not have any detectable effect on growth, in terms of size, of either the follicle or oocyte ($p>0.05$, figure 4.6). The culture medium however, was found to contain 0.3 mg/l (2.2 nM) of hypoxanthine (Sigma cell culture catalogue).

4.3.5. Stromal cells or collagen as a substrate for follicle growth

Stromal cells were used to form monolayers on which to culture follicles. The cell lines isolated were all fibroblastic in nature. Over the duration of culture, the cells plated onto the culture well surface and grew to confluence by the end of culture (day 5) (figure 4.7).

Culture on collagen resulted in larger follicles and oocytes by day 3 than those cultured on stromal cells ($p<0.05$) (figure 4.8). This difference in size persisted until the end of culture (day 5) ($p<0.01$) (figure 4.8).

Follicles cultured on both substrates appeared morphologically normal at the end of culture with an intact basement membrane and a clearly visible oocyte (figure 4.9). Follicles cultured on stromal tissue were surrounded by dense cellular growth (figure 4.9).

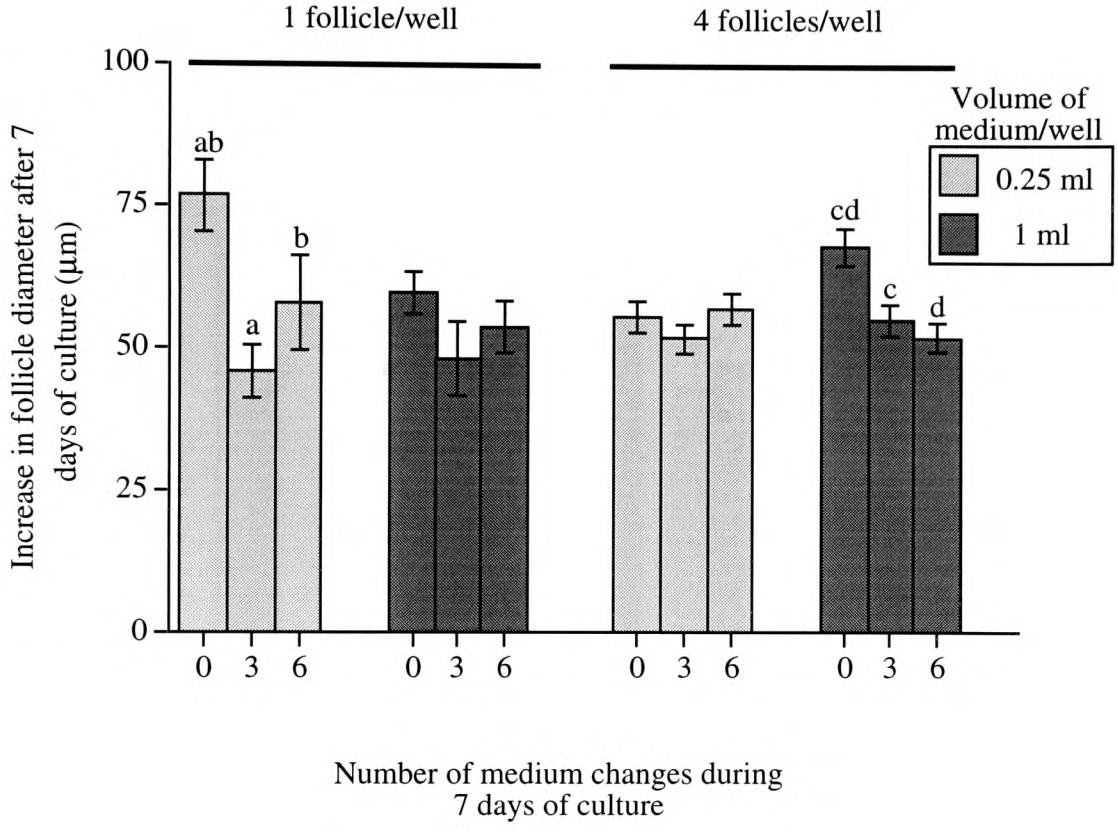


Figure 4.1.The effect of number of medium changes on the increase in follicle diameter after 7 days of culture. Results are mean ± s.e.m. Significant differences, as determined by a Student' t-test, between medium change regimes (within same follicle number and medium volume groups) are shown by the same letters ($p<0.05$).

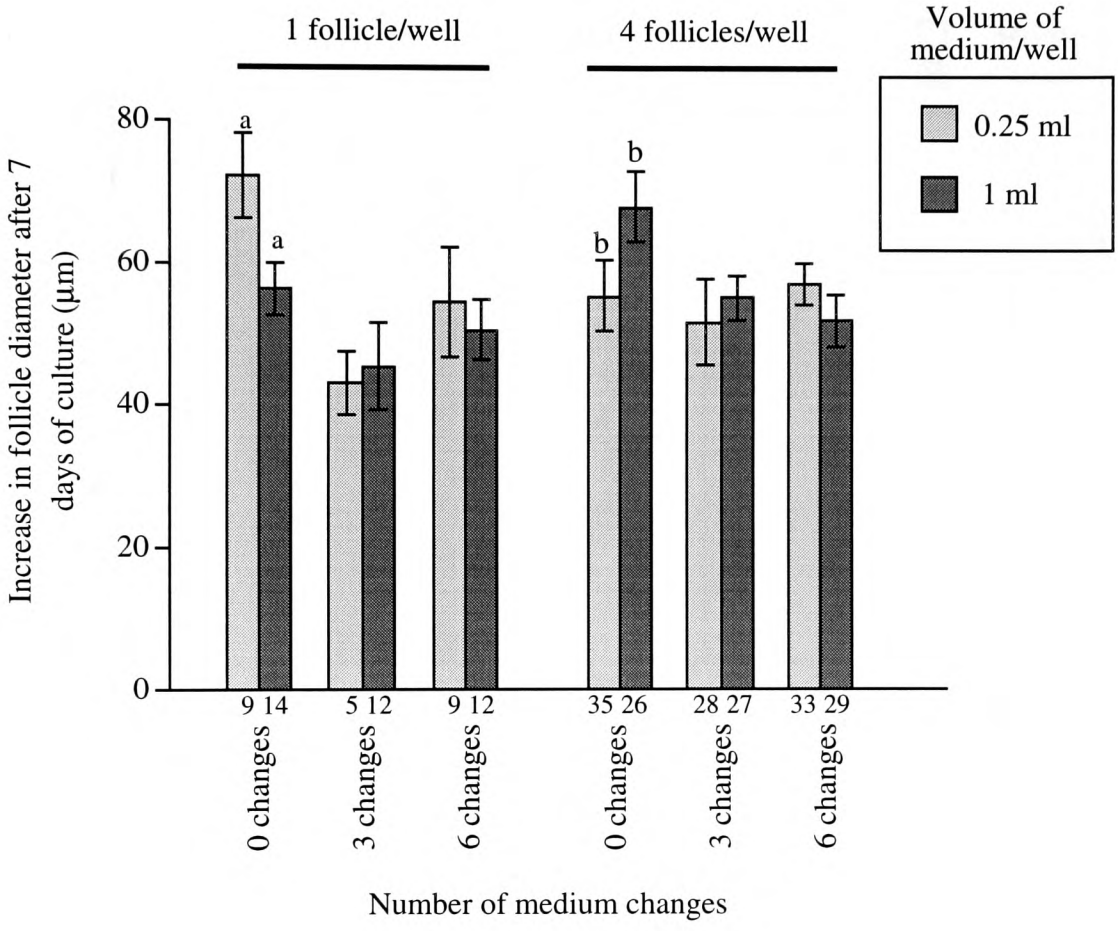


Figure 4.2. Histogram showing the effect of culture medium volume (0.25ml or 1ml) on the increase in follicle diameter after 7 of culture. Significant differences between different medium volume treatments within wells containing the same number of follicles and under the same medium changing regime, as determined by a Student's t-test, are shown by the same letters ($p<0.05$). Results are mean ± s.e.m.. The numbers below the x-axis represent the number of follicles per treatment.

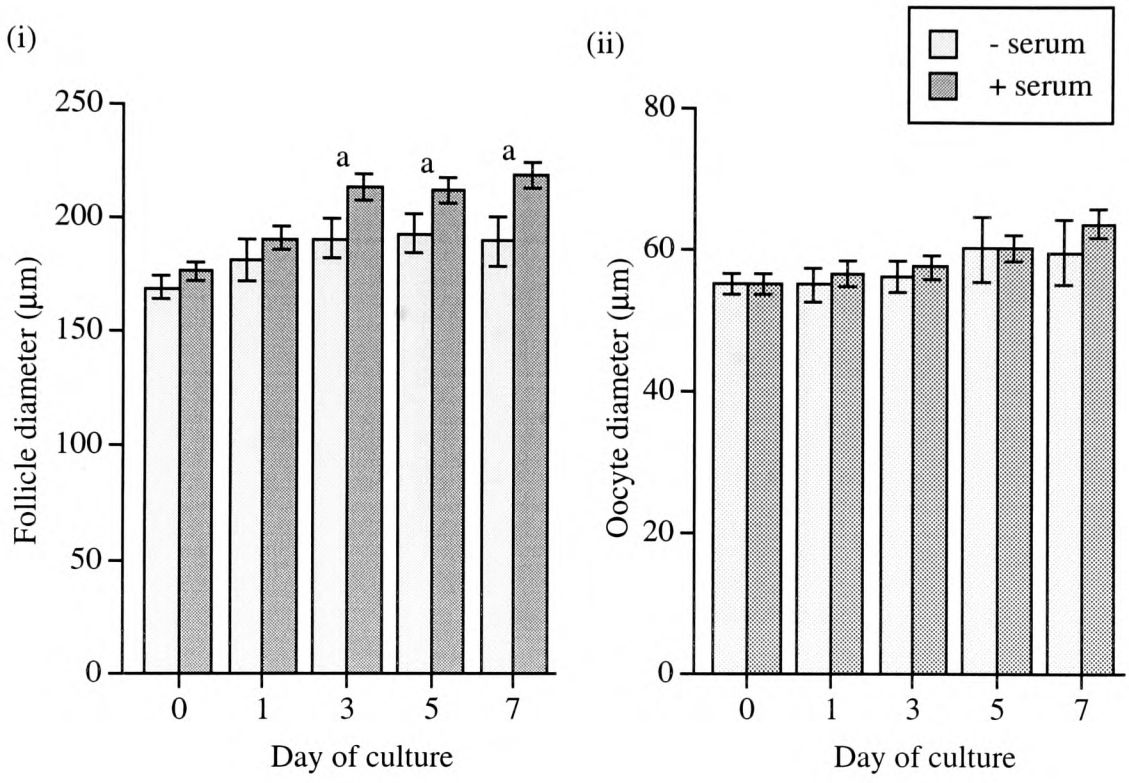


Figure 4.3. Histograms showing the effect of culture with (n=20 follicles) or without (n=21 follicles) heat inactivated foetal calf serum for 7 days on mean (i) follicle and (ii) oocyte diameter. [a]Significantly differences between follicle diameters ($p<0.05$) as determined by a Student's t-test. Results are mean ± s.e.m.

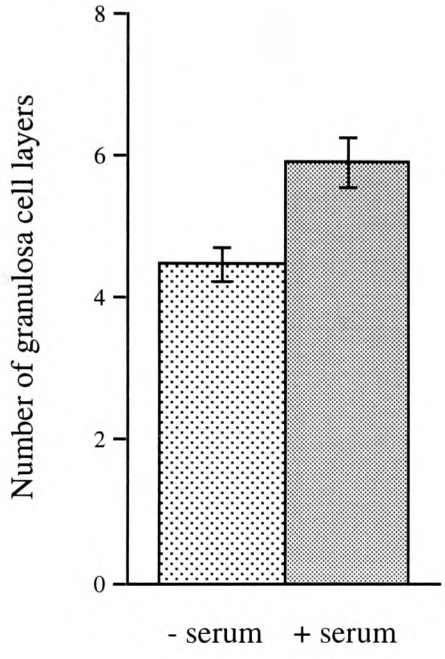


Figure 4.4. The effect of culture for 7 days in the absence (n = 19 follicles) or presence (n = 10 follicles) of heat inactivated foetal calf serum on the number of granulosa cell layers. Number of granulosa cell layers was determined in the oocyte nucleolus containing section. Results are mean ± s.e.m.

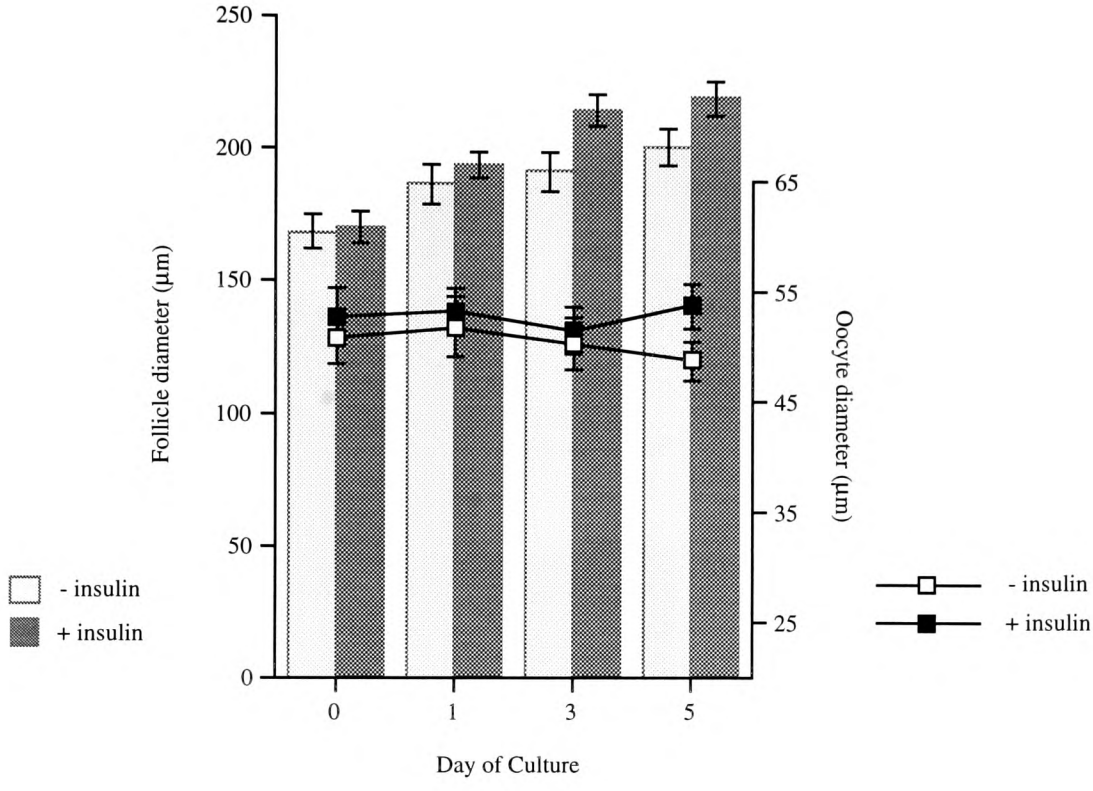


Figure 4.5. Graph showing the effect of insulin on follicle (histogram) and oocyte (line graph) diameters during culture for 5 days. Follicles were cultured with (n=15 follicles) or without (n=13 follicles) 5µg/ml of insulin in the culture medium. The addition of insulin increased follicle diameter on day 3 at levels approaching significance ($p<0.1$), and follicle and oocyte diameters on day 5 at levels approaching significance ($p<0.1$) as determined by a Student's t-test. Results are mean ± s.e.m.

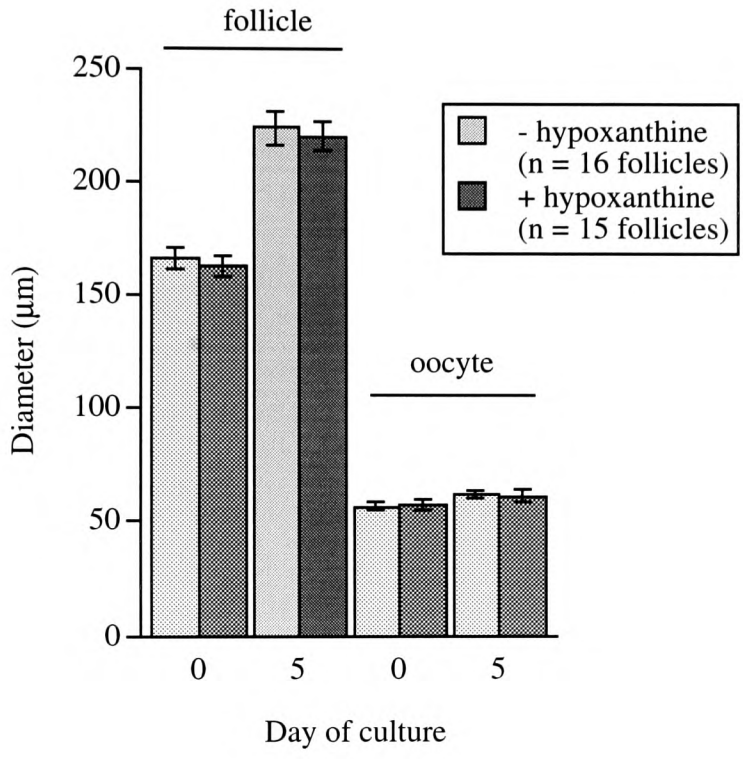
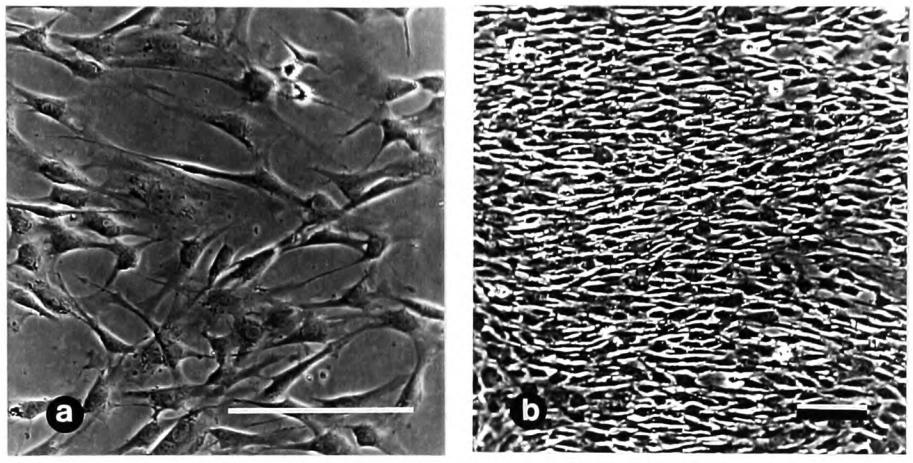
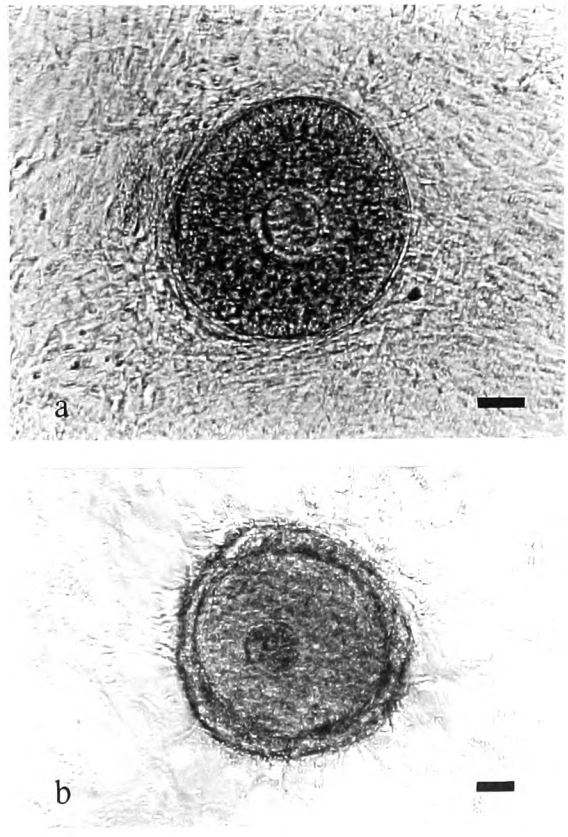


Figure 4.6. Histogram showing the effect of hypoxanthine on follicle and oocyte growth during culture for 5 days. No significant differences in follicle or oocyte diameter between treatments were observed after culture ($p<0.05$). Results are mean ± s.e.m.

Figure 4.7. Photomicrographs showing stromal cell monolayers on a. day 0 of follicle culture culture (1 day after adding cells to the well) and b. day 5 of culture. Bars represent 50 μm.

Figure 4.9. Photomicrographs showing follicles after 5 days of culture on a. stromal cell monolayer and b. collagen. Bars represent 50μm.


a
b


a
b

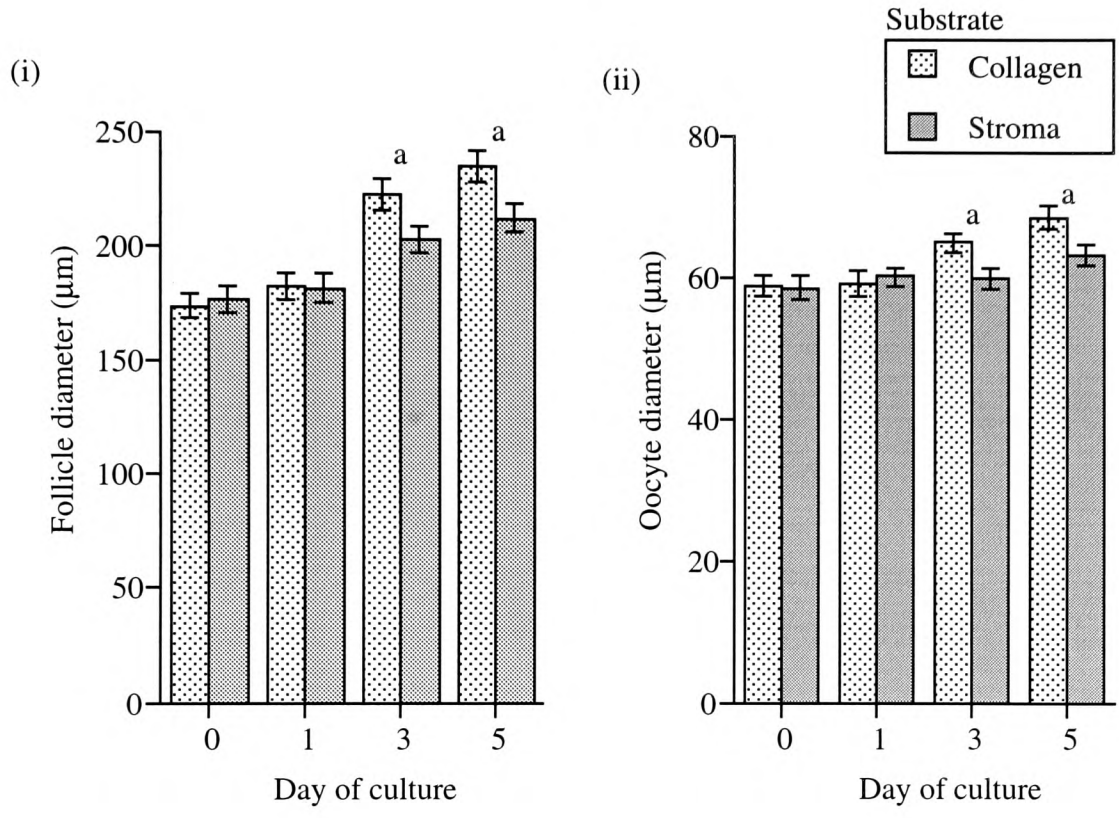


Figure 4.8. Histograms showing the effect of substrate on the growth of bovine preantral follicles cultured on either collagen (n=19 follicles) or stromal tissue monolayers (n=30 follicles) for 5 days. (i) Follicle and (ii) oocyte diameters were measured on days 0, 1, 3 and 5 of culture. [a]Culture on collagen resulted in significantly larger follicles and oocytes on days 3 and 5 ($p<0.05$) compared to culture on stromal cells as determined by a Student's t-test. Results are mean ± s.e.m.

Table 4.2. The effect of the number of medium changes and volume of medium on the increase in oocyte diameter following culture for 7 days.

	Increase in oocyte diameter following 7 days of culture (μm) $^{\text{s.e.m.}}_{\text{n}}$					
Volume of medium/well[b]	0.25 ml			1 ml		
Number of medium changes[a]	0	3	6	0	3	6
1 follicle/well	$6.53^{2.3}_{9}$	$1.96^{7.2}_{5}$	$5.44^{2.9}_{9}$	$3.85^{1.6}_{14}$	$-0.82^{4.7}_{12}$	$3.68^{2.2}_{12}$
4 follicles/well	$3.36^{1.4}_{35}$	$0.00^{2.0}_{28}$	$4.90^{1.5}_{33}$	$2.83^{2.1}_{26}$	$2.18^{2.1}_{27}$	$2.53^{2.1}_{29}$

[a]No significant effects of medium changes were detected between treatments containing the same number of follicles and volume of medium per well ($p>0.05$).

[b]No significant effect of volume of medium was detected between treatments containing the same number of follicles per well and under the same medium changing routines ($p>0.05$). Differences were determined by a Student's t-test.

Table 4.3. The effect of culturing 1 or 4 follicles per culture well on the mean increase in follicle and oocyte diameters during culture for 7 days.

	Number of medium changes	Increase in (i) follicle (ii) oocyte diameter after 7 days of culture (μm) ±s.e.m.			
		n	1 follicle/well [a]	n	4 follicles/well [a]
(i) Follicle	0	9	76.45 $^{6.1}$	26	67.75 $^{4.8}$
	3	5	45.57 $^{4.7}$	27	54.90 $^{3.1}$
	6	9	57.71 $^{8.2}$	29	51.70 $^{3.5}$
(ii) Oocyte	0	9	6.53 $^{2.3}$	26	2.83 $^{2.0}$
	3	5	1.96 $^{7.2}$	27	2.18 $^{2.1}$
	6	9	5.44 $^{2.9}$	29	2.53 $^{2.1}$

[a] No significant differences were detected between culturing as 1 or 4 follicles per well ($p>0.05$) under the same medium changing routines and volume of medium per follicle (0.25 ml/follicle) as determined by a Student's t-test.

Table 4.4. The effect of culture with or without heat inactivated foetal calf serum on the morphology follicles cultured for 7 days.

Measurement [a]	- serum[b] (n=19 follicles)	+ serum[b] (n=10 follicles)
Pyknotic cells	$2.42^{0.93}$	$2.90^{1.28}$
Follicle quality score	$1.42^{0.16}$	$1.50^{0.22}$
Oocyte quality score	$1.21^{0.20}$	$1.60^{0.22}$
Proportion with an antrum	$0.21^{0.10}$	$0.10^{0.10}$

[a] Measurements were made on the oocyte nucleolus containing section of the follicle.

[b] No significant differences were detected between culture with or without serum as determined by a Student's t-test. Results are mean ± s.e.m.

4.4. DISCUSSION

This series of experiments has been used to determine suitable conditions for the culture of bovine preantral follicles. (i) 0.25 ml of culture medium per follicle (ii) minimal number of medium changes (iii) the inclusion of serum and insulin in the culture medium and (iv) the use of collagen as a substrate were found to be appropriate for supporting bovine preantral follicle growth in vitro.

4.4.1. Effect of volume of culture medium, number of medium changes and the number of follicles per culture well on preantral follicle growth.

4.4.1.1. Number of follicles per well

The number of follicles per well had no effect on follicle growth when comparing cultures under the same medium changing routine and volume of medium per follicle. Previous studies in the mouse have detected inhibitory effects of culturing groups of follicles compared to individual follicles (Nayudu and Osborn, 1992, Spears et al., 1994). The comparisons made here between single or multiple follicle culture were based on the same volume of medium per follicle to reduce the possible effects of nutrient depletion or accumulation of waste products which may mask any true interfollicular dominance effect. It is also likely that differences in culture conditions and species could also account for these contrasting results. Interfollicular dominance effects of culturing follicles in groups may be caused by direct contact between follicles (Spears et al., 1994) and/or the production of follicle secreted factors (Nayudu and Osborn, 1992). Direct contact between follicles cultured here was avoided by positioning follicles equal distances apart at the start of culture which may also explain the absence of an effect of the number of follicles per well. In addition, it has been proposed that interfollicular dominance effects during preantral follicle

development in the mouse may act as a spacing mechanism within the ovary (Qvist et al., 1990, Nayudu and Osborn, 1992) which may not be as important in large mammals due to the larger ovary with proportionately more stromal tissue as suggested by Spears et al. (1994).

4.4.1.2. Volume of medium per well

The optimum volume of medium for follicle culture was 1ml for 4 follicles and 0.25ml for 1 follicle. Increasing or decreasing the amount of medium resulted in reduced follicle growth. By changing only half the volume of medium, it was hoped that a balance between removal of waste products, addition of fresh nutrient factors and retention of some follicle and oocyte produced factors could be obtained. Nevertheless, a significant effect of volume of medium was only observed if no changes were made during culture despite the fact that certain nutrients in the medium may be exhausted (e.g. insulin). However, the possibility that volume of medium is important when medium changes are made cannot be excluded as it may affect characteristics of the follicle other than size (e.g. oocyte developmental competence).

4.4.1.3. Changes of culture medium

Culture of follicles without changes of medium resulted in larger follicles by day 7 of culture when 4 follicles were cultured in 1ml or 1 follicle in 0.25ml. In contrast, murine preantral follicle culture showed that more medium changes resulted in enhanced follicle growth (Nayudu and Osborn, 1992). In our study, absence of medium changes may allow accumulation of follicle (e.g. activin, Findlay, 1993 (review)) and oocyte (e.g. GDF-9, Dong et al., 1996) produced factors the effects of which may have been masked by the use of FSH in the mouse experiment (Nayudu and Osborn, 1992). Alternatively, perturbation of the culture environment during medium changes (eg temperature, CO_2 concentration) may have effected follicle growth. By day 7 of culture, factors necessary for normal cellular growth (e.g. insulin), may be depleted (Hewlett, 1991) and it is likely that the beneficial effect of no

medium changes on follicle growth was a response to a transiently favourable environment. It is unlikely that follicle and oocyte development could be sustained any longer than examined here without replenishment of the medium.

4.4.1.4. Interaction between number of follicles per well, volume of medium and the number of changes

The optimum conditions for follicle growth depended on the number of follicles per well. For both 1 or 4 follicles, 0.25ml of medium per follicle proved to be the most suitable. The ideal medium volume per follicle is likely to provide a balance between a large enough pool for nutrient supply and waste product disposal yet in a volume small enough to allow accumulation of locally produced factors (see 4.4.1.3.). Medium changes were found to override any follicle number/medium volume interactions which may be due to either disturbance of culture conditions or addition or removal of growth regulatory factors (see 4.4.1.3.).

4.4.1.5 Summary

Volume of medium and number of medium changes affect follicular growth in vitro and optimum conditions depended on the number of follicles in the well. 0.25ml of medium per follicle gave the best follicle growth. Reducing or increasing the volume of medium per follicle depressed follicle growth rates possibly due to autocrine or paracrine effects which will complicate analysis of factors affecting follicular development. If the medium was changed, then the interaction between follicles/well and medium/well was not observed. For these reasons, experiments to examine the effects of factors on bovine preantral follicular development will use 0.25ml of medium per follicle with medium changes every second day. Reducing the number of medium changes and altering the volume of medium may prove useful for examining interfollicular effects in future studies.

4.4.2. The effect of serum on preantral follicle growth

Follicle growth was greater in the presence of serum than BSA. The effect of serum on follicle growth was due to an increase in the number of granulosa cell layers, indicating a stimulatory effect on granulosa cell proliferation. In contrast, during small preantral bovine follicle culture, serum stimulated follicle growth was a result of increases in granulosa cell size and not proliferation (Hulshof et al., 1995). Recent studies have shown that only small granulosa cells are proliferative and as granulosa cells increase in size their proliferative activity declines (Lederer et al., 1995). As an increase in the number of granulosa cell layers indicated proliferation had occurred, it was therefore unlikely that the increase in follicle diameter observed here was a result of increased granulosa cell size. These results also indicate the effect of serum may depend on the stage of follicle examined.

The factors in serum responsible for stimulation of follicle growth are not known but may be a combination of growth factors, hormones or nutrients. Gonadotrophins, known to stimulate follicle growth, were not present at detectable levels in the serum used here. However, IGF-I, which has a stimulatory action on follicle growth was present in the serum (60ng/ml) and in combination with other factors, may be responsible for the stimulation of follicle growth.

Although no effect of culture with or without serum on oocyte diameter was detected during culture, it is possible that it had other effects. It has been shown using cultured murine follicles that the absence of serum reduced oocyte developmental competence (Eppig et al., 1992). Developmental competence of oocytes cultured here was not assessed, nevertheless, no evidence of an effect of serum on oocyte morphology was detected. In addition, examination of other morphological characteristics showed that serum had no effect on follicle quality, number of pyknotic cells or proportion of follicles with an antrum.

In this study, foetal calf serum was heat inactivated before use (30 minutes at 56°C) which removed complement, thereby limiting the cytotoxic action of immunoglobulins (Freshney, 1987). Small polypeptides, such as growth factors or steroids should not be significantly damaged by heat inactivation (Freshney, 1987). Conversely, heat sensitive components, which may have important roles in follicular development, may be removed. There are no published comparisons between heat treated and untreated serum for in vitro follicle growth and few studies have been conducted in other culture systems, although one group have indicated heat inactivation of serum for in vitro bovine embryo development had little effect compared to untreated serum (Pinyopummintr and Bavister, 1994).

Development of a serum-free culture system for intact bovine preantral follicles would be hugely beneficial to studies of follicle development in vitro. This will require time consuming, repetitive and detailed work and is outwith the scope of this study. Serum-free culture systems used for granulosa cells (Buck and Schomberg, 1987) or murine granulosa cell-oocyte complexes (Eppig and O'Brien, 1996) have not been successfully applied to intact preantral follicle cultures (Nayudu and Osborn, 1992). It is likely that maintenance of the oocyte within a normal follicular structure requires a different balance of constituents and a number of additional factors (Nayudu and Osborn, 1992, Eppig et al., 1992). Until the factors essential for normal follicular development are identified, the success of serum-free follicle culture will be limited. Progress down this avenue has recently been achieved by the identification of an oocyte produced factor, growth differentiation factor-9 (GDF-9), required for follicle somatic cell differentiation (Dong et al., 1996). The identification of this factor and others in the future may overcome the deficiencies of serum free culture for in vitro growth of oocytes.

Completely defined culture systems would be difficult for intact follicle culture as (i) it uses material directly from the in vivo source and isolated follicles will contain undefined factors, especially if a fluid filled antrum is present, and the very

addition of follicles to culture medium makes the system undefined, (ii) BSA and fetuin, necessary supplements of serum-free medium for follicle growth in vitro are relatively undefined (Eppig et al., 1996, Hewlett, 1991) and (iii) factors in serum, which have not been identified, are necessary for normal follicle development (Eppig et al., 1992).

4.4.3. The effect of insulin on preantral follicle growth

Insulin promoted an increase in both follicle and oocyte diameters at a level approaching significance after 5 days of culture. The action of insulin promoting granulosa cell proliferation (Gong et al., 1993) may be responsible for the stimulation of follicle growth seen here.

In contrast to the results obtained here, addition of insulin to murine follicles resulted in smaller follicles by the end of culture (Spears et al., 1994). Species specific responses to insulin, differences in serum insulin (or other factor) levels and culture conditions could account for these conflicting results.

The quantity of insulin added to cell cultures is normally higher than the cells would be exposed to in vivo. The primary reason for this is that 90% of insulin is destroyed within 30 minutes at 37 ^{o}C (Hewlett, 1991). In this study, fresh aliquots of frozen insulin were used at the medium changes to minimise insulin degradation. In addition, serum may contain factors which may prolong the half life of insulin as binding proteins do for IGF-I (Guidice, 1992 (review))

IGF-I has actions similar to those of insulin (Giudice, 1992) and its presence in serum (60ng/ml) may have reduced the effect of insulin on follicle growth. It is not clear whether insulin affects follicle and oocyte growth by a different mechanism to IGF-I. However, insulin and IGF-I act via their own receptors (Gong et al., 1993, Carson et al., 1989) which suggests some independence of action. Alternatively, it is possible that IGF-I in the serum was inactive (possibly caused by the presence of binding proteins) and no interaction with insulin was observed.

4.4.4. The effect of additional hypoxanthine on preantral follicle growth

Hypoxanthine has been shown to have a beneficial effect on the preservation of oocyte granulosa cell cooperativity during follicle culture (Eppig and Downs, 1987) which is important for provision of nutrients to the oocyte (Heller et al., 1981) and therefore likely to affect oocyte growth. Addition of hypoxanthine to the culture medium did not affect the growth of either the follicle or oocyte in this study. The lack of effect of hypoxanthine on oocyte growth here may be due to the levels already present in the culture medium and serum and these are sufficient to maintain oocyte-granulosa cell cooperativity. However, previous studies using hypoxanthine free medium still did not detect any significant effect of hypoxanthine addition on oocyte growth (Carroll et al., 1991a, Figuiredo et al., 1994).

4.4.5. Comparison of stromal cells or collagen as a substrate for follicle growth

Maintenance of basement membrane integrity in combination with an increase in follicle size under both culture conditions indicates that some basement membrane remodelling has taken place in vitro. This is supported by recent studies showing that granulosa cells can produce collagen type IV, the collagen type forming the basement membrane, in vitro (Zhao and Luck, 1996).

The use of collagen as a substrate was more beneficial to follicle growth than stromal cell monolayers. Culture of follicles on stromal cells may provide extracellular matrix conditions similar to the ovary and allow the basement membrane integrity to be maintained as the follicle grows. Several practical problems are anticipated with the use of stromal cells (i) the monolayer becomes confluent before the follicle has stopped growing (ii) the large number of cells will rapidly deplete the medium of nutrients and will therefore require more frequent medium changes (iii)

analysis of the effects of factors on follicular development is made more complex (iv) the cultured stromal cells will be different from their in vivo counterparts.

The use of a hydrated collagen gel as a substrate for preantral follicle growth has proved successful in a number of species (mouse: Eppig and Telfer, 1993, Torrance et al., 1989, pig: Hirao et al 1994, cow: Figueiredo et al., 1995). Culture on collagen allows the follicles to attach, but not spread significantly and therefore avoid disruption of the follicular architecture. In this system, follicles were cultured on collagen. Culture in collagen provides additional 3 dimensional support and is more useful for follicles which have been isolated enzymatically in which damage to the basement membrane is likely. Culture on collagen allows the growth of the follicle and oocyte to be examined during culture and permits easy collection of the follicles for subsequent analysis.

This study indicated that culture on rats tail collagen maintained the follicle structure during culture as has been shown for non ovarian cells (Chambard et al., 1981). The component of the follicle exposed to the substrate are the theca/stromal cells where type I collagen has been localised to the extracellular matrix (Luck et al., 1995). Rats tail collagen used for this study, being type I collagen, is therefore a suitable substrate. Rats tails are a plentiful and economic source of collagen and were used as an alternative to the more costly collagen membranes. In addition, preformed collagen membranes have been dried which causes the 3-dimensional matrix to collapse and inhibits interaction with cells (Grinnell and Bennet, 1982).

In this study, culture on collagen resulted in the loss of thecal cells from the follicle surface. Theca cells, in addition to granulosa cells, produce components necessary for basement membrane maintenance (Luck et al., 1995). The basement membrane was intact and appeared morphologically normal after culture indicating adequate production of its components by the granulosa cells. The reason for detachment of thecal cells is not known but may be due to a deficiency in factors (e.g. fibronectin, laminins) affecting cell adhesion to the basement membrane (Hynes,

1993, Engvall, 1993). Of interest here is the inhibitory effect which insulin has on fibronectin production by granulosa cells (Skinner et al, 1985). The levels of insulin used during culture (5mg/ml) is within the dose range which caused maximal inhibition of fibronectin production by cultured granulosa cells (Skinner et al., 1985). In addition, fibronectin is sensitive to protease digestion (Ruoslahti et al., 1982). Proteases released by cellular disruption during follicle isolation may digest the fibronectin involved in adhesion of the surrounding theca/stromal tissue. Further work is required to determine why thecal cells are lost during culture.

4.4.6. A culture system for bovine preantral follicles

This series of experiments has been used to devise a basic culture system for the maintenance of bovine preantral follicles in vitro which will be used to examine the effects of other factors on bovine follicular development. The findings indicate that suitable conditions for the in vitro growth of bovine preantral follicles were: (i) 1 or 4 follicles per well, (ii) 0.25 ml of culture medium per follicle (iii) minimal number of medium changes (0 changes is unlikely to be suitable for sustained culture, so 3 changes will be used) (iv) the inclusion of serum and insulin in the culture medium and (v) the use of collagen as a substrate. It is likely that as follicles progress much beyond the preantral stage that their requirements will change and it will be necessary to re-evaluate the optimum conditions for more developed follicles.

CHAPTER 5: THE EFFECT OF FSH DOSE ON THE GROWTH OF ISOLATED BOVINE PREANTRAL FOLLICLES

5.1 INTRODUCTION

FSH is a principal regulator of follicular development. A wide range of studies have shown the effects of FSH during the final stages of follicular maturation (for reviews see: Fortune, 1994, Gougeon, 1996). Its effects during earlier follicular development are less clear. Receptors for FSH are present in early preantral bovine follicles (Wandji et al., 1992b). These receptors are likely to be functional as FSH stimulates granulosa cell proliferation and steroidogenesis in cultured early preantral follicles (cattle: Hulshof et al., 1995, hamster: Chiras and Greenwald, 1978, Roy and Greenwald, 1987).

Determination of a suitable FSH concentration in which to culture preantral follicles is difficult. Preantral follicle growth persists through a number of oestrus cycles where plasma FSH levels fluctuate between 10 and 15 ng/ml (Adams et al., 1992). The amount of FSH required during culture is likely to be much higher due to inactivation of FSH between medium changes. Furthermore, the levels of FSH in the circulation are suppressed by secretions of factors such as inhibin by the dominant follicle (Jong and Sharp, 1976) which may not promote optimum development of smaller follicles.

Culture of isolated preantral follicles from large mammals will allow the effects of FSH dosage on preantral follicle and oocyte development to be analysed. Extrapolation of the results may allow us to better understand follicular development in vivo and increase the success of assisted reproduction techniques such as superovulation.

The aim of this study is to use the system for the isolation and culture of bovine preantral follicles to: 1) define the optimum FSH dose for short term in vitro follicular development and 2) define the effects of FSH on follicular development in these stages of follicles.

5.2. METHODS

5.2.1. Follicle isolation and culture

Bovine ovaries were obtained from an abattoir at 25-30°C, rinsed with industrial methylated spirits and then soaked in PBS with 50mg/ml gentamicin (Life Technologies, Paisley, UK). Isolated large preantral/early antral follicles of approximately 180μm ± 20μm in diameter with several stromal/theca cell layers were isolated and cultured for 5 days as described in chapter 2.

5.2.2. Treatments

Two dose ranges of FSH were examined. Treatments common to both ranges were included which allowed comparisons between ranges to be made. Ovine FSH (Sigma) was used in both experiments. In range 1 (low FSH), FSH, at a concentration of either 0, 2.5, 25 or 250mIU/ml, was added to each well. 1mIU was equivalent to approximately 5.4ng FSH (source - Sigma Technical services). In range 2 (high FSH), FSH was added at a concentration of either 0, 250, 2500 or 25000mIU/ml to each well. The level of LH contamination of the preparation was low (<2%).

To determine granulosa cell proliferation, 0.185MBq of tritiated thymidine (Amersham Life Science, Buckinghamshire, UK.) was added to each culture well on day 4 for the penultimate 24 hours of culture. In addition, a control group of follicles were labelled with tritiated thymidine on days 1-2 of culture.

5.2.3. Histology

At the end of the culture period, follicles were fixed and sectioned for histology and autoradiography (chapter 2).

5.2.4. Collection of results

5.2.4.1. In culture

During the period of culture, follicle and oocyte diameters were measured as described in chapter 2. In a sample of follicles, the presence of a clearly visible antrum and its persistence during culture was examined. Follicles becoming degenerate during culture were excluded from the results.

5.2.4.2. Histology

Histological observations and measurements were made as described in chapter 2.

The number of granulosa cell layers was determined by counting the number of granulosa cells between the oocyte and the basement membrane in each section.

Silver grain clusters on the sections dipped in photographic emulsion indicated the incorporation of ^{3}H thymidine by the follicle in the final 24 hours of culture. The level of cellular proliferation was determined by counting the number of silver grain clusters in the follicle section containing the oocyte nucleolus. It is assumed that incorporation of ^{3}H thymidine indicated that cells have proliferated.

The frequency of the occurrence of antrum-like cavities was noted.

Oocyte quality was scored on a scale of 0 - 2:

0: Oocyte absent or severely misshapen with no germinal vesicle and obviously degenerate;

1: Misshapen oocyte, no germinal vesicle;

2: Morphologically normal oocyte, intact germinal vesicle;

5.2.4.3. Image analysis

Image analysis was performed on the follicles from the FSH range which contained the treatment found to give the best combination of follicle growth, oocyte growth and maintenance of oocyte morphology.

To ascertain the nature and differences in follicle growth between the different treatments, an image analysis system was used to measure the areas of the components of the follicle section. Images of the follicle section containing the oocyte nucleolus were analysed on an Apple Macintosh Power PC fitted with a Scion frame grabber card using the public domain NIH Image programme version 5.1 (U.S. National Institutes of Health, available from the internet by anonymous FTP from zippy.nimh.nih.gov). Follicle, oocyte and granulosa cell areas were measured using the area selection, density slicing and area measurement facilities (see appendix for macro programmes). From these measurements the area of the follicle not accounted for by the granulosa cells or the oocyte (intercellular spaces) was determined as:
Intercellular spaces area = follicle area - (oocyte area + total granulosa cell area).

5.2.5. Experimental design and statistical analysis

Four treatments were used in each FSH range. Limitations on the number of follicles which could be isolated from a single ovary resulted in partial replication of the experiment within each FSH range (typically 2 FSH doses in duplicate were set up per day). Combining results from the 2 ranges of FSH doses was only made if there were no significant differences between common treatments on follicle and oocyte growth.

Statistical analysis was performed using Minitab. Measurements were analysed on a per well basis to take into account the differences between wells and ovaries. These results were analysed initially by a one-way analysis of variance. Differences between treatments were then detected using a Students t-test. Results are

presented as the mean of the well averages for each treatment ± s.e.m. Frequency data was analysed per follicle and significant differences determined by a Chi-square test.

5.3. RESULTS

5.3.1. Follicle size

In all treatments, follicles increased in diameter during culture (figure 5.1.). The addition of FSH to the cultures further enhanced follicle diameter. Significant differences ($p<0.05$) in follicle diameter caused by the addition of FSH became apparent between days 3-5 of culture. No significant differences in follicle diameter between the concentrations of FSH used were detected ($p>0.05$).

5.3.2. Oocyte size

Oocytes increased in size over the 5 day culture period in all treatment groups (table 5.1.). High doses of FSH (25000mIU/ml) appeared to have an inhibitory effect on oocyte growth but this was not statistically significant ($p>0.05$). The largest increase in oocyte size was seen in the 25mIU/ml treatment group, although this was not significantly greater than the 0, 2.5, 250 or 2500mIU/ml treatment groups ($p>0.05$).

5.3.3. Oocyte morphology

Classification of oocyte quality indicated that high FSH concentrations may have an adverse effect oocyte development compared to the control without FSH at levels approaching significance ($0.1>p>0.05$) (figure 5.2.). In addition, the 250 and 2500mIU/ml treatment groups, whilst not displaying reduced oocyte growth, displayed some evidence of poorer quality oocytes. The addition of 2.5 or 25mIU/ml of FSH compared to the control group (0mIU/ml) did not significantly alter oocyte quality as determined here ($p>0.05$).

5.3.4. Follicle morphology

The reasons for increased growth of follicles on the addition of FSH and possible differences in the nature of the effect of FSH concentration were analysed. Granulosa cell proliferation (as determined by tritiated thymidine incorporation) was not significantly enhanced by the addition of FSH (figure 5.3.), indeed, high levels of FSH (≥2500mIU/ml) were seen to suppress proliferation levels compared to the lower FSH doses used. However, the differences in proliferation were not large enough to cause a change in the number of granulosa cell layers (table 5.2., $p>0.05$). It was unlikely that FSH stimulated follicle growth was a result of increase in the granulosa cell component of the follicle (figure 5.2., 5.3.). The effect of FSH on defined antral cavity formation (table 5.3., figure 5.4.) showed that FSH doses between 25 to 2500mIU/ml slightly (but not statistically significantly, $p>0.05$) increased the proportion of follicles with an antrum and that at high doses (25000mIU/ml), it had an inhibitory effect ($p<0.05$).

Examination of the persistence of antral cavities during culture (figure 5.5.) showed that if a defined antrum was present at isolation from the ovary, it is unlikely to be observed by day 3 of culture ($p<0.05$). This may indicate a redistribution of the antral cavity throughout the follicle.

Image analysis of the histological sections was carried out on the low range of FSH concentrations (figure 5.6.) as this contained the treatments which were found to stimulate maximal follicle (figure 5.1.) and oocyte growth (table 5.1.) whilst maintaining oocyte morphology (figure 5.2.). Image analysis was used to determine whether changes in follicle growth were due to an increase in the granulosa cell or the intercellular spaces components of the follicle (figure 5.6.). In agreement with the data on the number of granulosa cell layers and labelled cells, it was found that the low FSH doses did not alter total granulosa cell area in the sections examined (figure 5.6.(i)), $p>0.05$). However, when the total area of the intercellular spaces was

examined (figure 5.6.(ii)), it was found that FSH at a concentration of 2.5 or 250mIU/ml induced significant increases ($p<0.05$) over the control (0mIU/ml). An increase in intercellular spaces caused by the addition of 25mIU/ml FSH was observed, but this was not significantly greater than the control.

5.3.5. Morphological observations

The labelling of granulosa cells was often localised to the granulosa cell layers nearest the oocyte which was observed in all treatments (figure 5.7., table 5.4.). Equal levels of localisation were observed in follicles labelled on day 1-2 as on day 4-5 of culture (table 5.5., $p<0.05$). FSH dose did not significantly affect the percentage of follicles with oocyte localised proliferation (table 5.4., $p>0.05$). Localisation was never seen in sections where the oocyte was obviously degenerate. In the control treatment or at a low concentrations of FSH (2.5mIU/ml), proliferation in the granulosa cell layer nearest the oocyte (figure 5.8.(i))was significantly greater ($p<0.05$) than the 250, 2500 or 25000mIU/ml FSH groups. Three granulosa cell layers away from the oocyte (figure 5.8.(ii)), only the highest FSH doses (2500, 25000mIU/ml) resulted in lowered levels of proliferation ($p<0.05$).

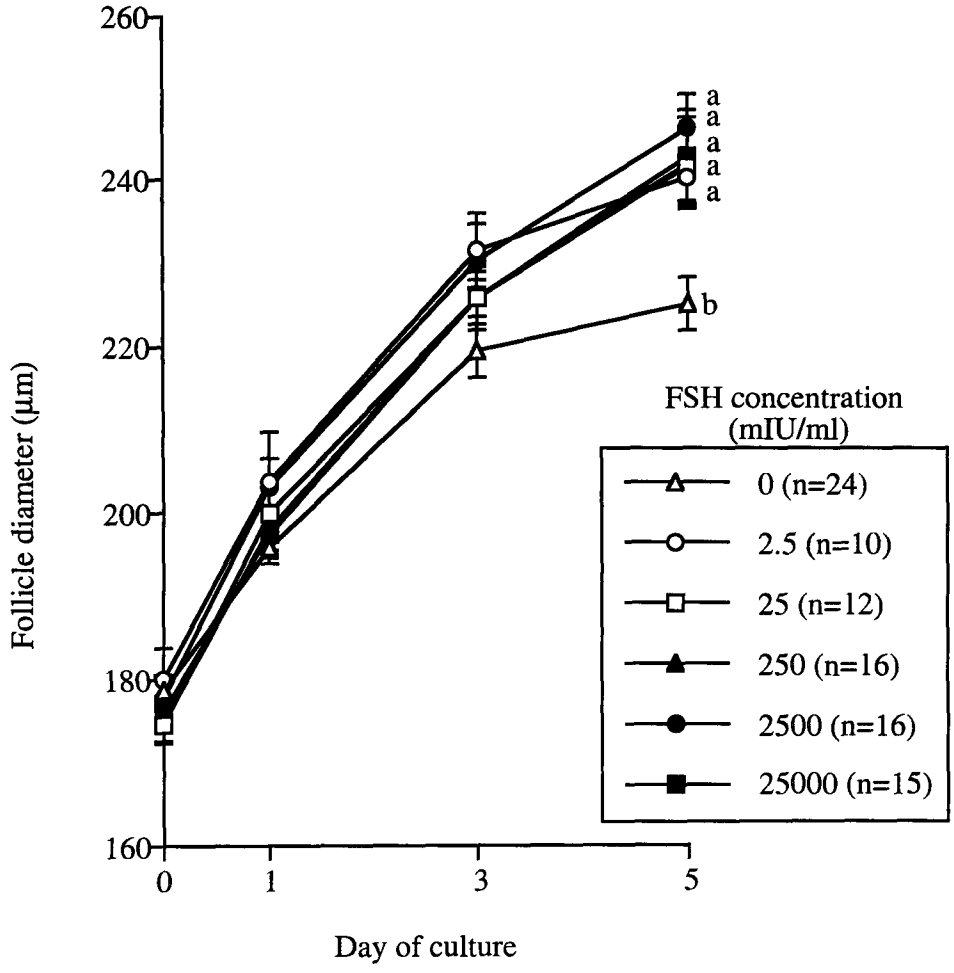


Figure 5.1. The effect of FSH (0, 2.5, 25, 250, 2500 or 25000 mIU/ml) on follicle diameter during culture. Results presented are the mean follicle diameter ± s.e.m. Significant differences ($p<0.05$) as determined by the Student's t-test are shown by different letters. n=number of culture wells (4 follicles per well at the start of culture).

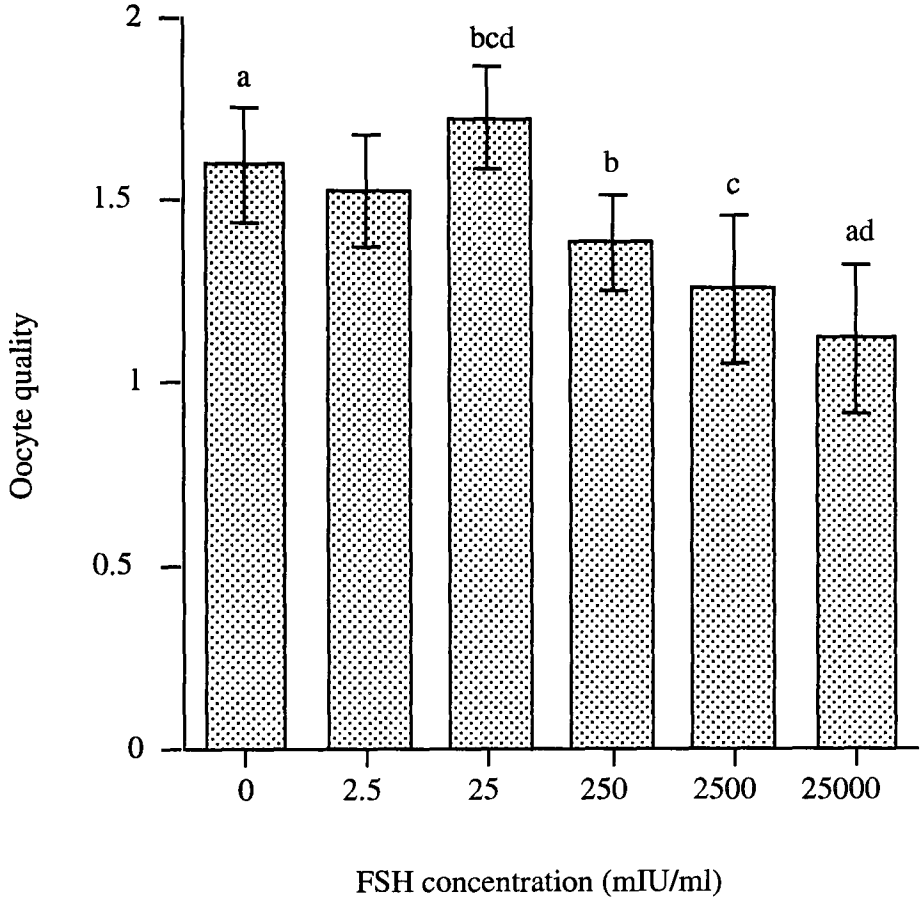


Figure 5.2. The effect of 0 (n=11), 2.5 (n=13), 25 (n=13), 250 (n=19), 2500 (n=11) or 25000mIU/ml (n=16) of FSH on oocyte quality of follicles cultured for 5 days. Results are mean ± s.e.m. Significant differences between treatments are shown by d ($p<0.05$), possible differences between treatments ($p<0.1$) are shown by a,b and c, as determined by a Student's t-test.

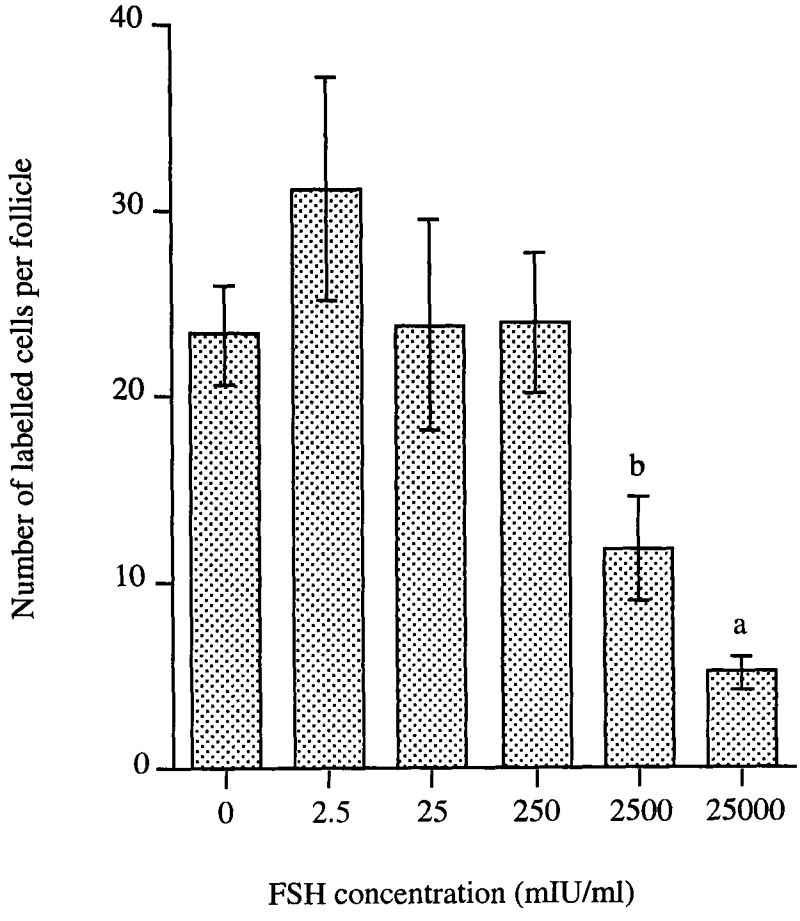
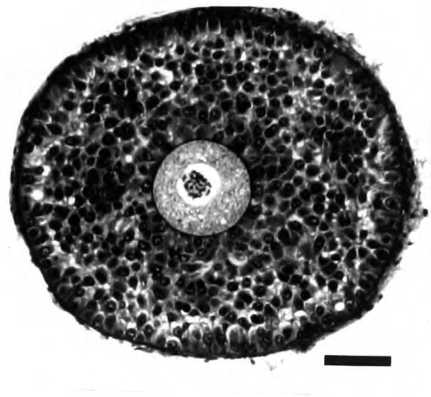
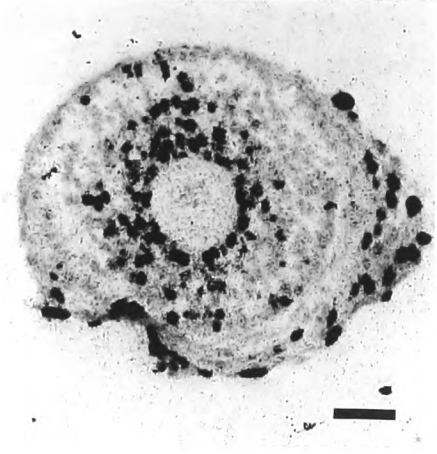


Figure 5.3. The effect of 0 (n=11), 2.5 (n=13), 25 (n=13), 250 (n=19), 2500 (n=11), 25000mIU/ml (n=16) of FSH on the number of cells labelled per follicle (as determined by tritiated thymidine incorporation) on days 4-5 of culture. Results are means ± s.e.m. Significant differences were determined by a Student's t-test; a was significantly smaller ($p<0.05$) than all other treatments, b was significantly smaller than 0, 2.5 or 250mIU/ml ($p<0.05$) and possibly the 25mIU/ml treatment group ($p<0.1$).

Figure 5.4. Semi-thin section of a 5 day cultured preantral follicle stained with toluidine blue. An antrum was not visible. Bar represents 50μm

Figure 5.7. Section of a 5 day cultured preantral follicle labelled with tritiated thymidine and stained with haemotoxylin and eosin. Proliferation was often localiosed to the granulosa cell layers surrounding the oocyte. Bar represents 50 μm.





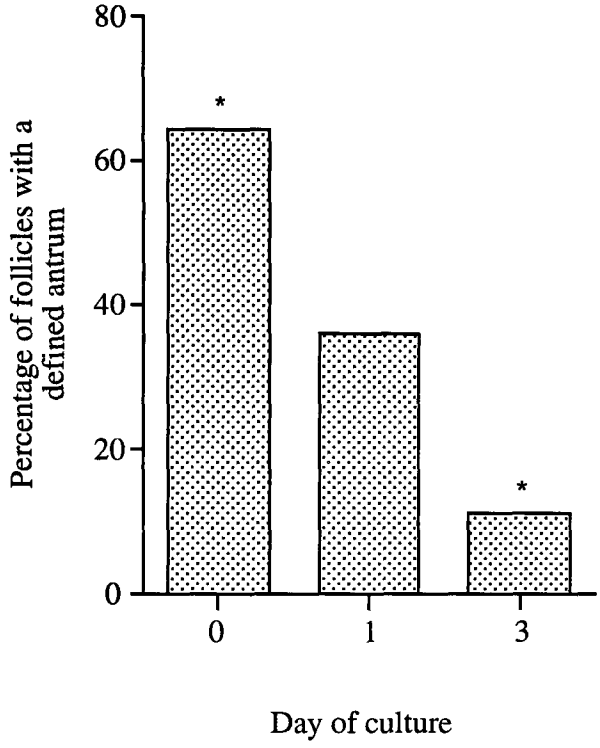


Figure 5.5. The effect of culture on the presence of a visible antral cavity during culture of follicles. * Significant differences between days, as determined by a Chisquare test ($p<0.05$). n=28 follicles cultured with 25mIU/ml FSH.

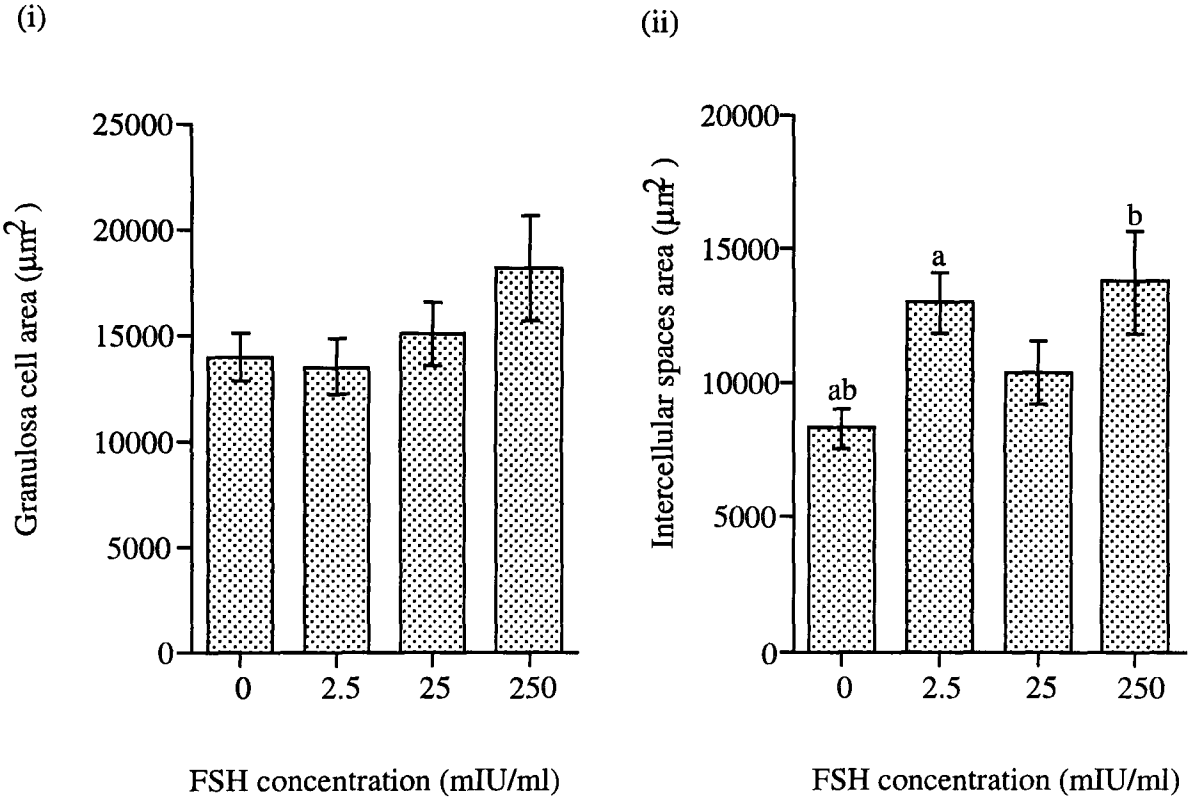


Figure 5.6. The effect of 0 (n=6), 2.5 (n=12), 25 (n=13) or 250mIU/ml (n=6) of FSH on the (i) total area of granulosa cells or (ii) area of intercellular spaces in the oocyte nucleolus containing section of follicles cultured for 5 days. Results are mean ± s.e.m. n=number of culture wells (4 follicles per well at the initiation of culture). Significant differences bteween treatments ($p<0.05$), as determined by a Student's t-test, are shown by the same letters.

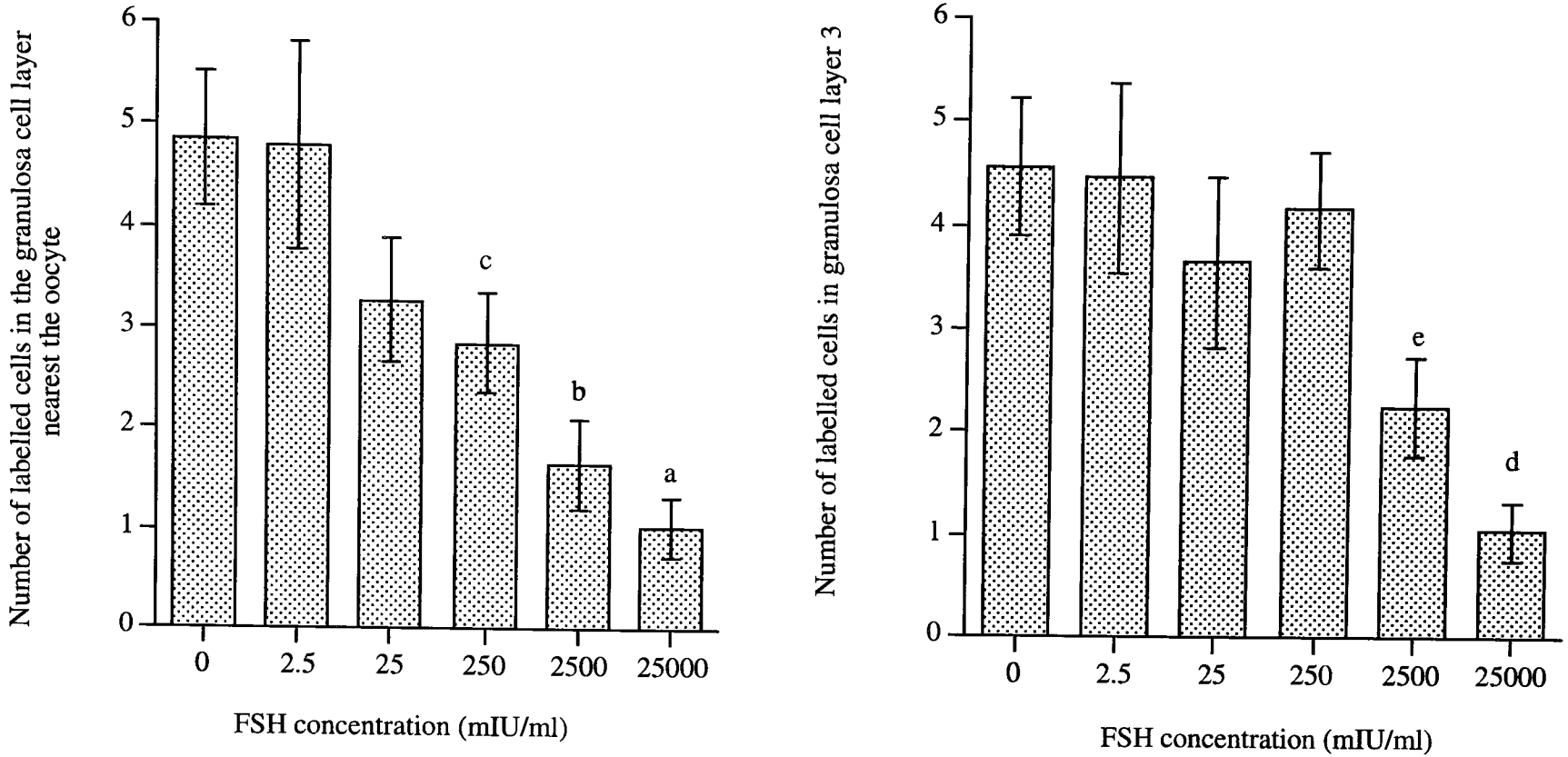


Figure 5.8. The effect of 0 (n=11), 2.5 (n=13), 25 (n=13), 250 (n=19), 2500 (n=11) or 25000mIU/ml (n=16) of FSH on the number of granulosa cells labelled in (i) the layer nearest the oocyte and (ii) the 3rd layer distant from the oocyte on days 4-5 of culture. n=number of culture wells (4 follicles per well at the initiation of culture). Results are means ± s.e.m. Significant differences were determined by a Student's t-test; In graph (i) a was significantly smaller than the 0, 2.5, 25 and 250mIU/ml treatment groups ($p<0.05$), b was significantly smaller than the 0, 2.5 and 25mIU/ml ($p<0.05$) and possibly smaller than the 250mIU/ml ($p<0.1$) treatment groups, c was significantly smaller than the 0 and 2.5mIU/ml treatment groups ($p<0.05$), in graph (ii) d was significantly smaller than the 0, 2.5, 25, 250 and 2500mIU/ml treatment groups ($p<0.05$), e was significantly smaller than the 0, 2.5 and 250mIU/ml ($p<0.05$). No significant differences were detected between the 0, 2.5, 25 and 250mIU/ml FSH treatment groups.

Table 5.1. The effect of FSH dose on the growth of bovine oocytes during preantral follicle culture for 5 days.

FSH concentration (mIU/ml)	no. of wells	Oocyte diameter (μm) (s.e.m.)[a]		Diameter change[a] (μm) (s.e.m.)
		Day 0	Day 5	
0	24	58.76 (0.68)	63.58 (1.16)	+ 4.82 (0.87)
2.5	10	61.67 (0.78)	65.64 (1.59)	+ 3.97 (1.82)
25	12	59.91 (0.92)	68.04 (1.11)	+ 8.13 (0.94)
250	16	58.13 (1.08)	62.76 (0.96)	+ 4.63 (0.74)
2500	16	57.85 (1.05)	62.20 (0.95)	+ 4.35 (1.13)
25000	15	59.65 (1.00)	62.41 (1.80)	+ 2.76 (1.76)

[a] Analysis of variance did not detect any significant differences in oocyte diameter on either day 0, day 5 of culture or in the diameter change between different FSH concentrations. Results are mean ± s.e.m.

Table 5.2. The effect of FSH concentration on the number of granulosa cell layers in follicles cultured for 5 days.

FSH concentration (mIU/ml)	no. of wells	Average number of granulosa cell layers per follicle[a]	s.e.m.
0	11	5.1	0.24
2.5	13	5.2	0.22
25	13	4.9	0.28
250	19	5.1	0.22
2500	11	5.2	0.20
25000	16	4.8	0.22

[a] No significant differences between rows were detected as determined by the Student's t-test

Table 5.3. The effect of FSH on the percentage of 5 day cultured follicles with an antrum-like cavity as determined from histological sections

FSH concentration (mIU/ml)	n[b]	% follicles with an antrum-like cavity[a]	s.e.m.
0	11	13.64	6.6
2.5	13	11.54	8.3
25	13	22.44[1]	6.4
250	19	21.05[2]	5.4
2500	11	19.70[3]	7.0
25000	14	4.17[123]	2.9

[a]Significant differences between treatments are represented by the same superscripts (1, 2 $p<0.05$), possible differences are shown by 3 ($p<0.1$) as determined by a Chi square test.

[b]n is the number of culture wells (4 follicles per well at the start of culture)

Table 5.4. The percentage of follicles exhibiting localisation of granulosa cell proliferation around the oocyte on days 4-5 of culture

FSH concentration (mIU/ml)	n	% follicles[a] with granulosa cell proliferation localised to the oocyte[b]
0	20	40.0
2.5	33	42.4
25	20	65.0
250	15	53.3

[a] only follicles with morphologically normal oocytes were included. No significant differences ($p>0.05$) between rows, as determined by Chi square analysis, were detected.

[b] granulosa cell proliferation was determined by tritiated thymidine incorporation.

n=number of follicles

Table 5.5. Localisation of granulosa cell proliferation to the cells nearest the oocyte at different stages of culture[a]

Day of culture	n[b]	Percentage of follicles with proliferation localised to granulosa cells near the oocyte[c]
2	35	40
5	15	40

[a]Follicles were cultured for up to 5 days in the absence of FSH

[b]n=number of follicles

[c]No significant difference was found between the percentages of localisation on the 2 days examined.

5.4. DISCUSSION

The effect of increasing doses of FSH on the in vitro growth of isolated bovine preantral follicles was examined.

Addition of FSH to the culture medium resulted in larger follicles than in the control groups following 5 days of culture. That FSH stimulates bovine preantral follicle development has previously been demonstrated both in vivo (Monniaux et al., 1984) and recently confirmed in vitro (Nuttinck et al., 1996, Wandji et al., 1996a). The effect of FSH on follicle growth was not further enhanced by increasing FSH dose. During murine preantral follicle growth in vitro, steroid production increased dose dependently with FSH to the highest dose tested (2000mIU/ml), but follicle growth reached a threshold at 100mIU/ml (Nayudu and Osborn, 1992). It is likely that the maximum response of other follicular characteristics to FSH is different and the optimum concentration for bovine preantral follicle development need not be that which results in maximum growth alone.

The effect of FSH on follicular development has been the subject of much debate and the precise state of differentiation of the granulosa cells examined and FSH dose may account for the conflicting opinions in the literature. The nature of FSH induced growth of the preantral follicles cultured here did not appear to be due to an increase in either the number or size of granulosa cells as neither total granulosa cell area, number of granulosa cell layers or proliferating cells were significantly enhanced on addition of FSH. In previous studies, the effect of FSH on small bovine preantral follicles (<100μm) found either increased granulosa cell proliferation (Wandji et al., 1996a) or increased granulosa cell size (Nuttinck et al., 1996). In this study, although FSH was not present at detectable levels in the foetal calf serum, other factors stimulating proliferation (e.g. IGF-I (present at 60ng/ml in the serum)) used may have masked any effect of FSH on granulosa cell proliferation.

A reduction in granulosa cell proliferation at the higher doses of FSH (≥2500mIU/ml) was observed in our study which may be attributed to the effects of FSH on granulosa cell differentiation. It has previously been shown that high doses of FSH result in differentiation of rat granulosa cells (Lederer et al., 1995) and this is characterised by increased steroidogenesis and almost complete loss of mitotic activity (Lederer et al., 1995).

FSH is required for antrum formation in rodent follicles (Boland et al., 1993, Roy and Greenwald 1989, Nayudu and Osborn, 1993). FSH did not increase the number of follicles exhibiting an antrum at the end of culture in this study. However, FSH was found to significantly increase intercellular spacing within the granulosa cell layers. This spacing may be analogous to antrum development, which would be expected in follicles of this size in vivo, but that the culture conditions are deficient in stimulating coalescence of these spaces to form a morphologically discrete antrum. Further evidence for this was shown by a reduction in the number of follicles showing an antrum on day 3 of culture compared to day 0. Studies of the postnatal bovine ovary have shown that at 240μm, 80% of follicles exhibit a discrete antrum (Lussier et al., 1987, Monniaux et al., 1984) of around 5000μm^2 (Monniaux et al., 1984). Cultured bovine follicles did not show comparable levels of antral cavity formation at this stage, but the area of intercellular spacing more than accounted for the expected antral size. However, it has been shown that in vivo administration of PMSG (FSH like activity) delays antrum formation in follicles >180μm so that it occurred in larger follicles compared to those without treatment (Monniaux et al., 1984). The delay in antrum formation was thought to be due to high proliferation of granulosa cells which would predominate over secretion of antral fluid. This was unlikely to account for the lack of antrum formation here as (i) proliferation was not enhanced by FSH (ii) the percentage of follicles with an antrum at 240μm in vivo was still much higher than after culture (approximately 60% in vivo compared to around 20% after culture).

Foetal calf serum was included in the bovine follicle cultures used here and it is likely that FSH interacts with components of the serum such as IGF-1 (Giudice, 1992). The inclusion of serum for the in vitro growth of intact murine follicles was thought to be necessary for maintenance of follicle structure and steroidogenesis (Nayudu and Osborn, 1992) and production of competent oocytes (Eppig et al., 1992). BSA has been used to replace serum, although this also contains a number of undefined components. All the factors required for normal follicle development have yet to be identified but it is hoped that in the future a completely defined system may be used which would allow more detailed studies follicular development.

Oocytes increased in size over the culture period. Although FSH stimulated follicle growth, it did not significantly effect oocyte growth as measured by diameter. Follicles which had been grown in the presence of FSH therefore had a larger follicle to oocyte ratio than those cultured without FSH. Follicle and oocyte growth can be dissociated (Eppig et al., 1992), therefore conditions which may enhance follicle growth may not promote increased oocyte development.

The effect of enhanced follicle growth may influence oocyte development in a way which did not manifest itself by an increase in oocyte size. Scoring of oocyte quality showed that higher doses of FSH, whilst not significantly compromising oocyte diameter, resulted in poorer quality oocytes. Since oocytes themselves are not directly responsive to FSH, this inhibitory effect must be mediated via the granulosa cells. FSH induced degeneration of oocytes in small bovine preantral follicles has been suggested to be a result of inappropriate granulosa cell differentiation perhaps causing reduced gap junction communication between the oocyte and surrounding granulosa cells (Nuttinck et al., 1996) which may be important for oocyte nutrient provision (Eppig, 1991).

Granulosa cell proliferation was often concentrated in the layers nearest the oocyte. The localisation effect occurred in all the FSH classes examined independently of dose. The effect was unlikely to be a culture artefact as it was

observed at equal frequencies on days 1-2 of culture compared with days 4-5 of culture. Secretion of an oocyte produced granulosa cell proliferating factor(s) has been demonstrated in the mouse (Vanderhyden et al., 1992) and it is possible that bovine oocytes may produce a similar factor. Here, only follicles containing a morphologically normal oocyte exhibited localised granulosa cell proliferation suggesting a regulatory role of the oocyte rather than innate proliferative ability. The intensity of proliferation in the granulosa cell layer nearest the oocyte was reduced at FSH doses >25mIU/ml whereas only the highest dose reduced proliferation of granulosa cells more distant from the oocyte. These results show a difference in responsiveness of granulosa cells to FSH depending on their position relative to the oocyte which may be involved in granulosa cell differentiation into mural and cumulus granulosa cells. In addition, the higher FSH sensitivity of the granulosa cells nearest the oocyte may enable the oocyte to be acutely responsive to its hormonal environment which may ultimately affect the fate of the follicle.

In this study we have used an isolation and culture system to examine the effect of a hormonal factor on the in vitro growth of intact bovine preantral follicles. As ovarian follicles develop, their responses to hormonal stimulation changes. An aim for future research will be to examine different stages of bovine follicles and determine the effects of other factors on follicular development.

CHAPTER 6. THE EFFECT OF FSH ON BOVINE PREANTRAL AND EARLY ANTRAL FOLLICLE DEVELOPMENT IN VITRO.

6.1. INTRODUCTION

Current methods for increasing the production of competent oocytes for assisted reproduction rely on the small population of large antral or preovulatory follicles. Isolation of many immature, growing preantral follicles and their culture to a stage where the oocytes could be fertilised would allow an increase in oocyte production. Initial studies have concentrated on murine preantral follicles (Daniel et al., 1989, Eppig and Schroeder, 1989, Roy and Greenwald, 1989, Torrance et al., 1989, Nayudu and Osborn, 1992), where an abundant supply of growing preantral follicles can be readily isolated, grown to a stage where the oocyte becomes competent to resume meiosis, be fertilised and the embryos develop to term (Eppig and Schroeder, 1989).

The development of culture systems for the growth of oocytes from larger mammals is at an early stage. Isolation and culture of livestock preantral follicles has been limited in success (for review see Telfer, 1996). A larger ovary, less densely packed follicles, fibrous stromal tissue, follicular size and rate of follicular growth present problems for adapting the techniques developed in rodents to livestock species.

The isolation and culture of preantral ovarian follicles also provides a convenient model to study follicular development. Culture of isolated granulosa cells has provided much valuable information into many aspects of follicular development. However, it is becoming clear that the oocyte has an important role in controlling several somatic cell functions such as proliferation, steroidogenesis, cumulus cell

expansion (reviewed by Buccione et al., 1990a). In whole ovarian follicle culture, the 3-dimensional communication between the oocyte and its surrounding somatic cells is maintained thus allowing studies of in vitro follicular development of greater physiologically relevance.

Initial studies on the isolation and culture of bovine preantral follicles (Jewgenow and Pitra 1991, Taha and Schellander 1992, Hulshof et al., 1992) examined the growth of small preantral follicles <100μm in diameter. In these studies, mechanical and enzymatic methods enabled the rapid release of large numbers of small follicles. Complete in vitro growth of small preantral bovine follicles to preovulatory stages is an ambitious aim since preantral development is lengthy (4-5 months) (Lussier et al., 1987, Betteridge et al., 1989) and prolonged culture would be necessary if developmentally competent oocytes are to be obtained. An alternative approach would be to utilise the population of large preantral follicles entering the rapid antral phase of development (early antral to preovulatory development takes around 1-2 months) (Lussier et al., 1987, Betteridge et al., 1989). Isolation of these follicles by mechanical or enzymatic methods as used for smaller follicles may damage the structure of the larger preantral follicles, therefore a different isolation technique which maintains follicular morphology is required.

The gonadotrophin, follicle stimulating hormone (FSH), is a principal regulator of follicle growth. FSH is known to be involved in granulosa cell proliferation, differentiation, follicular steroidogenesis, antrum formation, follicle selection and prevention of atresia (Greenwald and Roy, 1994). The precise nature of the role of FSH during preantral follicle development is unclear. Using hypophysectomised sheep it has been shown that preantral follicle development may proceed independently of gonadotrophic support but as antrum formation progresses, follicles become increasingly dependent on the continuing support of FSH for their survival (Dufour et al., 1979). However, studies in the hamster have shown that

although not essential, FSH can enhance preantral follicle growth (Roy and Greenwald, 1986).

The work presented here describes a simple microdissection technique which isolates preantral to early antral ovarian follicles which are intact and are morphologically normal. The effect of the addition of FSH to bovine ovarian follicle development as they progress from the preantral to the early antral stage, becoming more responsive to FSH, was examined.

6.2. METHODS

6.2.1. Follicle isolation and culture

Bovine ovaries were obtained from an abattoir and follicles were isolated as described in chapter 2. Isolated follicles of approximately 90 to 270 μm in diameter with several stromal/theca cell layers, an intact basement membrane, evenly pigmented granulosa cell layer and oocyte were selected for culture (figures 6.1.a, b, c). Selected follicles were divided into 3 size classes: preantral (approximately 90 μm to 150 μm), large preantral/early antral (approximately 150 μm to 210 μm) and antral (approximately 210 μm to 270 μm). Isolated follicles were cultured on collagen as described in chapter 2.

6.2.2. Treatments

Ovine FSH (Sigma) at a concentration of either 0 or 25 mIU/ml (approximately 135 ng/ml) was added to each well. The concentration of FSH used was previously found to give optimal levels of granulosa cell proliferation and maximal stimulation of large preantral/early antral follicle growth (Ralph et al, 1995b, chapter 5). The level of LH contamination of the preparation was low (<2 %).

0.185 MBq of tritiated thymidine (Amersham Life Science, Buckinghamshire, UK.) was added to each culture well on the penultimate day of culture to determine granulosa cell proliferation .

6.2.3. Histology

At the end of the culture period, follicles were dislodged from the substrate, washed in fresh medium, fixed and processed for autoradiography (see chapter 2).

A small number of follicles were embedded in plastic and processed for semi-thin sectioning and staining (chapter 2)

6.2.4. Collection of results

6.2.4.1. In culture

During the period of culture, follicle and oocyte diameters were measured (chapter 2). The average follicle diameter was calculated and used for analysis.

6.2.4.2. Histology

Histological observations and measurements, using a light microscope (chapter 2), were made on the section containing the oocyte nucleolus or largest cross section of the oocyte if the nucleolus was absent. The number of granulosa cell layers was determined by counting the number of granulosa cells between the oocyte and the basement membrane in each section.

Silver grain clusters on the sections dipped in photographic emulsion indicated the incorporation of ^{3}H thymidine by the follicle in the final 24 hours of culture. The level of cellular proliferation was determined by counting the number of silver grain clusters as a proportion of the estimated total cell number in the section. It is assumed that incorporation of ^{3}H thymidine indicated that cells have proliferated.

6.2.4.3. Image analysis

To ascertain the nature of follicle growth between the different follicle classes with or without the addition of FSH, an image analysis system was used to measure the areas of the components of the follicle section. Images of the follicle section containing the oocyte nucleolus were analysed on an Apple Macintosh Power PC fitted with a Scion frame grabber card using the public domain NIH Image programme version 5.1 (U.S. National Institutes of Health, available form the internet by anonymous FTP from zippy.nimh.nih.gov). Follicle, oocyte and granulosa cell areas were measured using the area selection, density slicing and area measurement facilities (see appendix for list of macro commands). From these

measurements the area of the follicle not accounted for by the granulosa cells or the oocyte (intercellular spaces) was determined as:

Intercellular spaces area = follicle area - (oocyte area + total granulosa cell area).

The area of the granulosa cells was also used to make an approximation of the labelling index. An estimate of the number of granulosa cells in the section was made by dividing the area of the granulosa cells by an estimated granulosa cell area of 28.26 μm^2 (assuming that granulosa cells are near spherical with a radius of 3 μm). The proportion of cells having undergone cellular division (i.e. incorporating ^3H thymidine) could then be calculated.

6.2.5. Experimental design and statistical analysis

The experiment was based on a 2 by 3 factorial design. The follicles placed in a culture well came from the same ovary. Limitations on the number of follicles which could be isolated from a single ovary resulted in partial replication of the experiment within each ovary.

Statistical analysis was performed using Minitab. Analysis of data was made on a per well basis to minimise the variation between follicles from the same ovary. Measurements made during culture were analysed as the increase in follicle or oocyte diameter between days 0 and 5 of culture using a Student's t-test. Histology measurement data was analysed initially by a one-way analysis of variance. Differences between treatments were then detected using a Students t-test and the results presented as the mean of each treatment ± s.e.m. Frequency data was analysed by a Chi-square test.

6.3. RESULTS

6.3.1. Follicle growth

In all treatments, follicles increased in diameter during culture regardless of the size at the initiation of culture (figure 6.2.a). Follicles in culture whose basement membrane ruptured, became flattened and spread over the collagen surface were regarded as degenerate and excluded from the results (figure 6.1.d).

The addition of FSH to the cultures enhanced follicle diameter in antral and large preantral/early antral class follicles ($p<0.05$), but not that of the preantral follicles ($p>0.05$). Differences in follicle diameter in the FSH treatment group became apparent between days 3-5 of culture. The increase in follicle size caused by the addition of FSH was greater for antral follicles than for large preantral/early antral follicles.

6.3.2. Oocyte growth

In all follicle classes and treatments, oocytes increased in size over the 5 day culture period (figure 6.2.b). No significant effect of FSH on oocyte growth in terms of an increase in size was observed ($p>0.05$). Of the follicles sectioned for histology, between 17 and 35% of oocytes were obviously degenerate at the end of culture (table 6.3.). Neither the addition of FSH or the class of follicle affected the proportion of follicles becoming degenerate during culture.

6.3.3. Image analysis

The nature of the effect of FSH on follicle growth and morphology was determined by image analysis.

The addition of FSH did not significantly affect the granulosa cell area in any of the follicle classes examined ($p>0.05$)(figure 6.3.a). This is confirmed by analysis of the number of granulosa cell layers in the follicles where the addition of

FSH did not have a significant influence on granulosa cell layer numbers in a follicle class ($p>0.05$)(figure 6.4.).

Most of the increase in follicle size caused by FSH treatment in the large follicles may be explained by significant increase in the area of spaces found in the non oocyte compartment of the follicle ($p<0.05$)(figure 6.3.b). No such differences were detected in the medium and small follicle groups ($p>0.05$). It is likely that this method of measurement is not sensitive enough to detect the slightly smaller differences in follicle growth in the large preantral/early antral group on the addition of FSH.

6.3.4. Morphological observations

6.3.4.1. Granulosa cell proliferation

^{3}H thymidine incorporation was used as an indication of the levels of cellular proliferation in the penultimate 24 hours of culture. Granulosa cell proliferation was observed in all follicle classes and treatments (table 6.1.). No significant differences in the estimated labelling index between follicle classes or different concentrations of FSH were detected at the end of culture ($p>0.05$).

The labelling of granulosa cells was often localised to the granulosa cell layers nearest the oocyte. This localisation was observed in all follicle classes and treatments (figure 6.5.a). However, localisation was never seen in sections where the oocyte was obviously degenerate (figure 6.5.b). This phenomenon was present in all follicle classes and treatments (table 6.2.). The percentage of follicles showing granulosa cell proliferation localisation was significantly smaller on the addition of FSH only in the large preantral/early antral follicle class.

6.3.4.2.General follicle morphology

Most of the follicles appeared healthy after culture with an intact basement membrane, apparently normal oocyte held in meiotic arrest, close oocyte-granulosa cell association and few pyknotic cells (figure 6.6.).

Defined antrum formation was rarely observed in the medium and large class follicles at the end of culture when they were at a size where one would be expected in the comparable in vivo grown follicles. If small antral cavities were apparent at the initiation of culture, they were rarely observed to persist for the duration of culture.

Thecal cells (or their stromal tissue precursors), were present at the initiation of culture. A defined thecal cell layer was rarely observed by day 5 (figure 6.6.). This apparent loss of the thecal cell layer was unlikely to be due to inhibition of growth in culture as the thecal cells strongly incorporated ^{3}H thymidine, indicating that they were proliferating.

The granulosa cells in freshly isolated follicles were organised into distinct layers. In contrast, granulosa cells in cultured follicles appeared less organised.

Figure 6.1. Freshly microdissected bovine (a) preantral follicle, (b) early antral follicle (c) histological section of an antral follicle (toluidine blue). Note the presence of a small antrum (A), theca/stroma tissue (T), intact basement membrane (BM), evenly pigmented granulosa cells (G) and oocyte (O). (d) Attachment in culture resulted in basement membrane rupture, granulosa cell spreading (S) and eventual release of oocyte (arrow), these follicles were considered abnormal and their results were excluded from analysis. Bars represents 50μm.

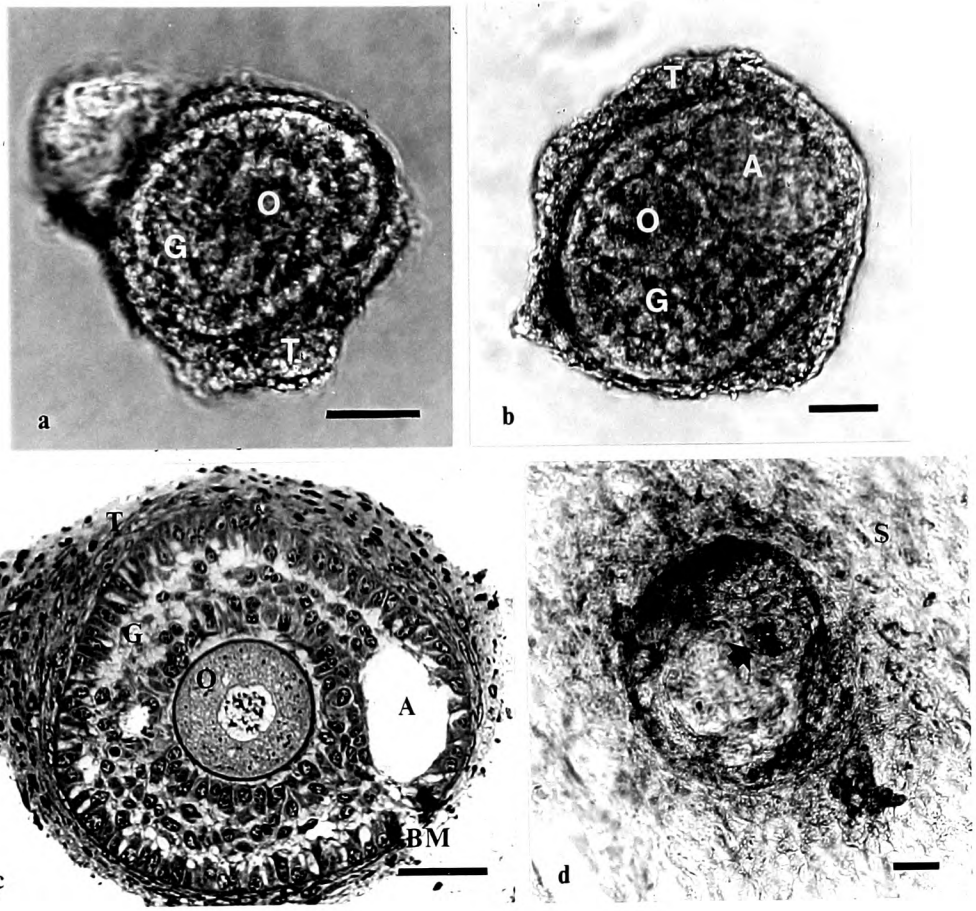
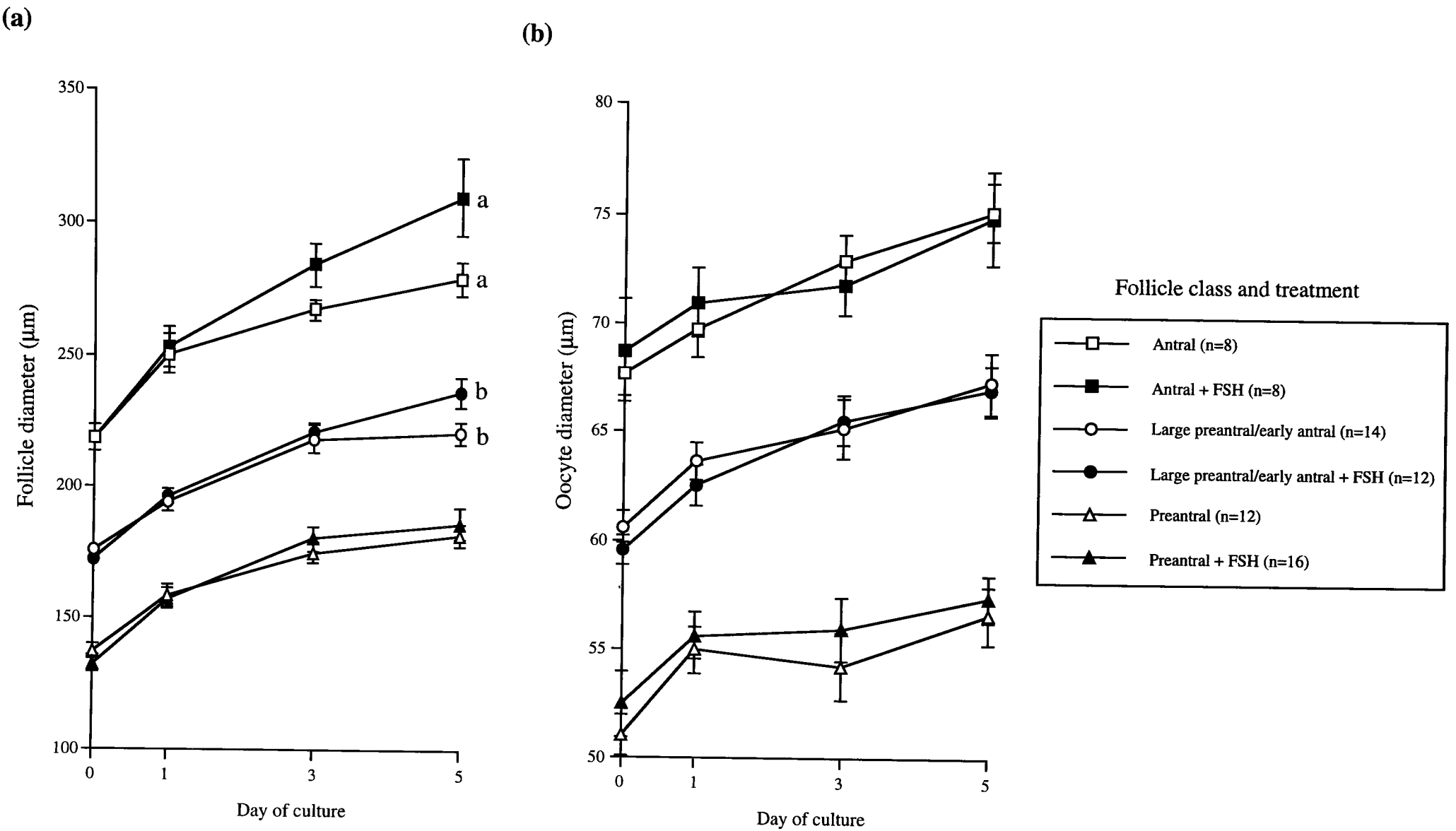

O
G
T
a
T
A
O
G
b
T
G
O
A
BM
c
S
d

Figure 6.2. Graph of follicle (a) and oocyte (b) growth profiles during the culture of preantral, large preantral/early antral and antral isolated follicles. Follicles were measured in culture on days 0, 1, 3 and 5. Culture was continued for 5 days in the presence or absence of 25mIU oFSH. Results are mean ± s.e.m. , n = number of culture wells (4 follicles per well). [ab] Significant differences in the increase in follicle or oocyte diameter over the 5 day culture period between culture with or without FSH within the same follicle size class are shown by the same letters ($p<0.05$). Differences were determined by a Student's t-test.


(a)
(b)
Follicle diameter (µm)
350
300
250
200
150
100
a
a
b
b
0
1
3
5
Day of culture
Oocyte diameter (µm)
80
75
70
65
60
55
50
0
1
3
5
Day of culture
Follicle class and treatment
Antral (n=8)
Antral + FSH (n=8)
Large preantral/early antral (n=14)
Large preantral/early antral + FSH (n=12)
Preantral (n=12)
Preantral + FSH (n=16)

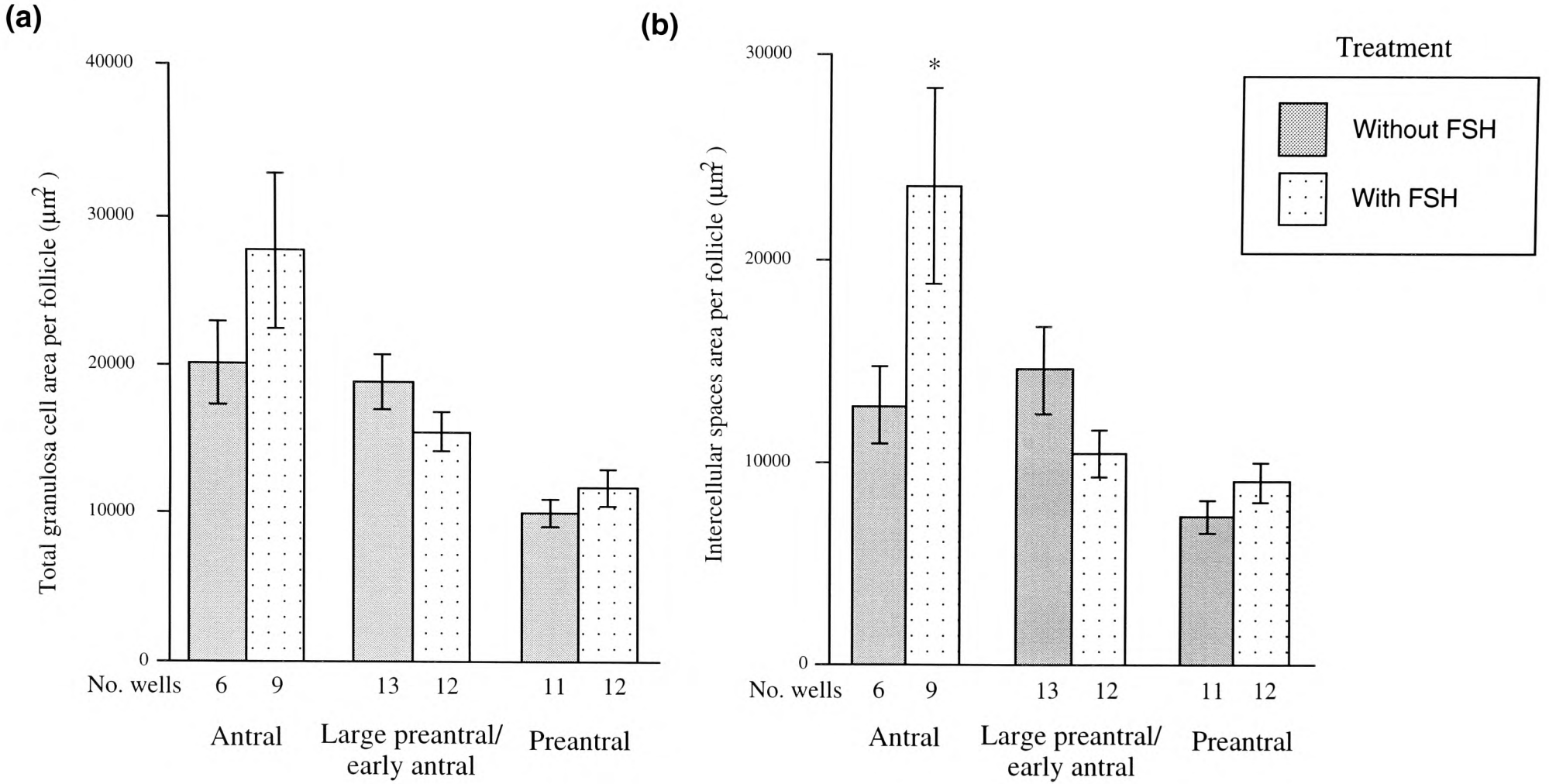


Figure 6.3. Histogram showing (a) Total granulosa cell area, (b) the intercellular spaces within the granulosa cell layers in the nucleolus containing section of preantral, large preantral/early antral and antral follicles cultured with/without 25mIU oFSH for 5 days as determined by image analysis. Results shown are the averages ± s.e.m. * Significant differences within follicle size classes were determined by a Student's t-test ($p<0.05$).

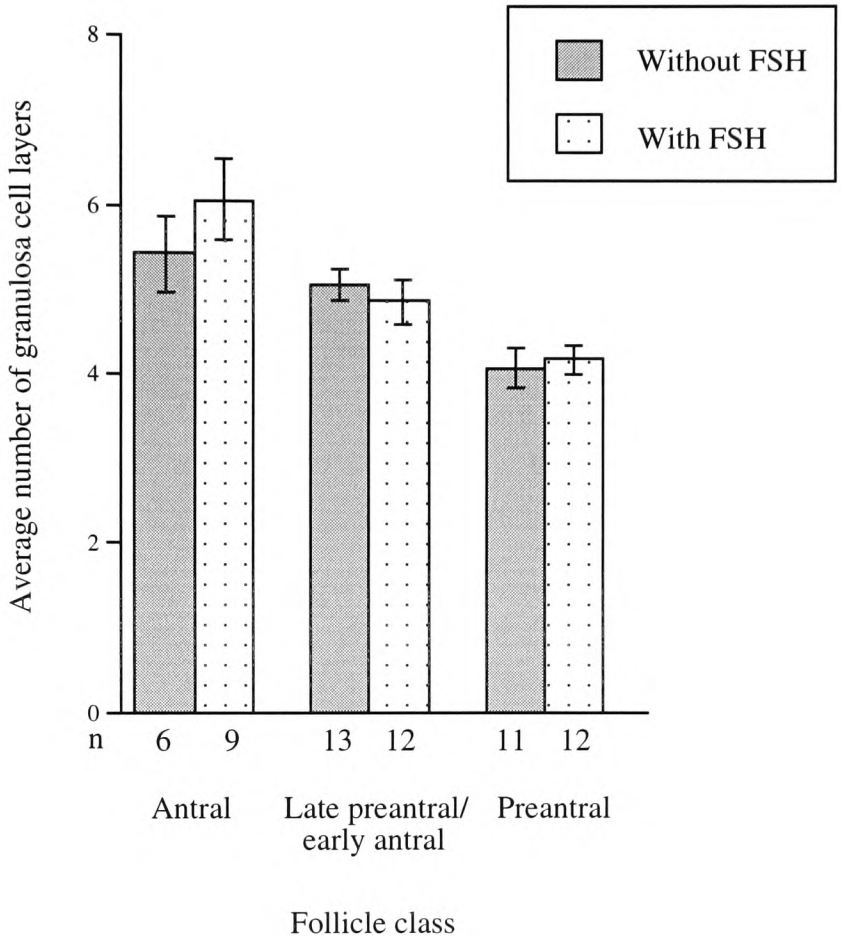
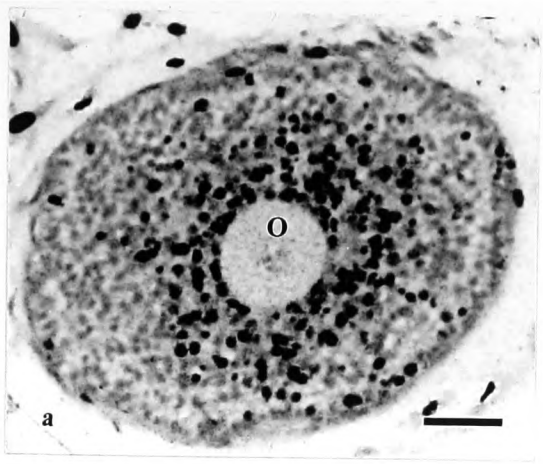
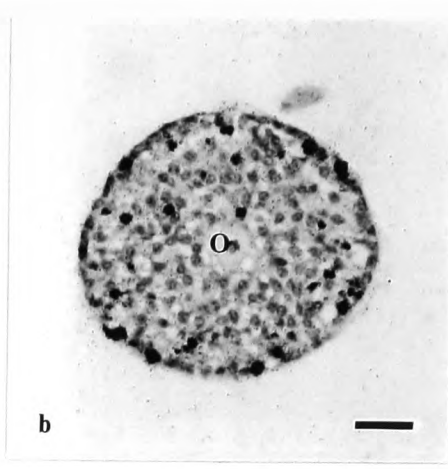


Figure 6.4. Histogram showing the number of granulosa cell layers present in preantral, large preantral/early antral and antral follicles cultured for 5 days with/without 25mIU FSH. Results are the mean number of granulosa cell layers per follicle ±s.e.m. No significant differences ($p>0.05$) as determined by the Student's t-test were detected within follicle classes on the addition of FSH. n=number of wells (4 follicles per well).

Figure 6.5. Localisation of proliferation of granulosa cells (as determined by tritiated thymidine incorporation and autoradiography) to the layers nearest the oocyte (O) occurred if (a) a morphologically normal oocyte was present but not (b) if the oocyte was degenerate. Bars represent 50 μm.

Figure 6.6. Semi-thin section of a healthy follicle cultured for 5 days in the presence of FSH. Oocytes were maintained in meiotic arrest (O), close oocyte granulosa contact, basement membrane was intact (BM), few pyknotic granulosa cells were present. A defined theca layer was absent. Bar represents 50 μm.


O
a


O
b

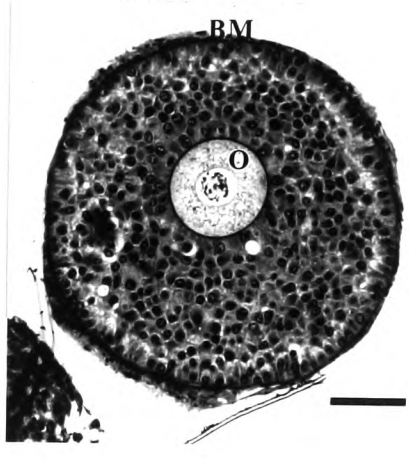

BM
O

Table 6.1. Estimated labelling indices of cultured follicles

Follicle class	Treatment	no. wells	Labelling index (%) [a]	s.e.m [b]
Early antral	- FSH	6	4.55	1.27
	+ FSH	8	4.50	0.77
Large Preantral/ early antral	- FSH	13	4.69	0.65
	+ FSH	12	4.14	0.80
Preantral	- FSH	11	3.85	0.76
	+ FSH	12	3.96	0.73

[a] the proportion of cells incorporating tritiated thymidine in the penultimate 24hrs of culture.

[b] no significant differences between different groups were detected using a Student's t-test.

Table 6.2. The percentage of oocytes exhibiting localisation of granulosa cell proliferation

Follicle class	Treatment	% follicles[a] with granulosa cell proliferation localised to the oocyte[b]
Early antral	- FSH	65
	+ FSH	59
Large Preantral/ early antral	- FSH	76[c]
	+ FSH	34[c]
Preantral	- FSH	40
	+ FSH	29

[a] only follicles with morphologically normal oocytes were included

[b] granulosa cell proliferation was determined by tritiated thymidine incorporation

[c] significant differences ($p<0.05$) between culture with/without FSH in the same follicle class, as determined by Chi square analysis, are show by the same superscripts. Analysis was performed on the frequency data and presented here as percentages.

Table 6.3. The percentage of follicles exhibiting morphologically normal oocytes

Follicle class	Treatment	% follicles with normal oocyte [a]
Early antral	- FSH	83
	+ FSH	79
Large Preantral/ early antral	- FSH	71
	+ FSH	78
Preantral	- FSH	80
	+ FSH	65

[a] Chi square analysis did not detect any differences between treatments or groups. Analysis was performed on the frequency data and presented here as percentages.

6.4. DISCUSSION

The effect of FSH during the in vitro development of isolated bovine follicles as they progress from the preantral to early antral stage was examined.

The initiation of antral development coincides with entry into the rapid growth phase of the follicle and final preparation of the oocyte for ovulation. In contrast, development of early preantral follicles to the antral stage is lengthy, taking many months in vivo (4-5 months in sheep, Cahill and Mauléon, 1980) and prolonged culture will be required if appreciable follicular development is to be realised. Large preantral to early antral follicles (130-285 μm diameter) grow to preovulatory size (3.6-8.6 mm) in around 40 days in cattle (Lussier et al., 1987). In cattle the antrum forms in follicles between 115 μm (where 10% have early antral cavities) to 280 μm (where 90 % have an antrum) (Monniaux et al., 1984). In this study follicles were divided into 3 classes (preantral, 90–150 μm, large preantral/early antral, 150–210 μm and antral, 210–270 μm) which would allow the effect of FSH on follicles during antrum formation to be examined.

FSH stimulated an increase in the size of the large preantral/early antral and antral follicles, but not that of the preantral follicles. It seems likely that these increases reflected an accumulation of intercellular fluid. Stimulation of follicle growth in these classes coincides with an increased ability to bind FSH (Wandji et al., 1992b). As FSH did not affect the growth of the oocyte, the increase in follicle size was a result of a change in the granulosa cells or intercellular spaces. There was no evidence of an increase in number, area or labelling index of the granulosa cells. A significant rise in intercellular spacing of antral follicles was detected. It is likely that the methodology employed was either not sensitive enough to detect the smaller FSH induced changes in growth of the large preantral/early antral follicles or that shrinkage of the spaces had occurred during processing for histology. In addition, follicles which had a small defined cavity at the initiation of culture were found not to have antral cavities at the end of culture. It is probable that the precocious antral cavity was redistributed throughout

the follicle during culture. Increases in intercellular spacing were also seen in cultured murine ovarian follicles when the antrum failed to form (Boland et al., 1993) and these follicles increased in size at the same rate as those follicles in which an antrum did form (Boland et al., 1993) supporting the idea that intercellular spaces within the granulosa cell layers are analogous to the antrum.

In the follicles cultured here, antrum formation was rarely observed. The mechanisms behind the antrum formation are not well understood (Gosden et al., 1988) and species differences may exist. In the mouse, follicles develop antral cavities when a theca layer is present either in culture (Nayudu and Osborn, 1992) or after transplantation under the kidney capsule (Telfer et al., 1990). By contrast, antrum formation did not occur in culture after loss of theca cells during follicle isolation with collagenase (Telfer et al., 1990). In the pig, but not the mouse, antrum formation did occur in the absence of theca cells when follicles were cultured with the additional structural support of encapsulation in a collagen gel (Hirao et al., 1994, Telfer et al., 1990). It appears that the role(s) of theca cells in antrum formation may either be through the production of factors which modify granulosa cell function (Bendell et al., 1988) and/or influences on physical constraint.

In this study, several layers of theca/stromal tissue were present at isolation but during culture these cells, whilst proliferating strongly, did not adhere to the basement membrane and failed to develop into a multilayered structure. The absence of a defined theca layer may explain why an antrum did not form during culture.

In cattle, oocytes acquire the competence to mature and proceed to metaphase II at diameters >110 μm (Fair et al., 1995b). Follicles isolated here contained oocytes in mid growth phase (50 – 70 μm in diameter) and therefore have considerable growth and development to achieve before becoming competent to resume meiosis.

FSH did not affect the growth of the oocyte. In the developing follicle FSH receptors are expressed exclusively by the granulosa cells (Camp et al., 1991) and although oocytes are not themselves directly responsive to FSH, the stimulatory effect of

FSH on follicle growth was not passed onto the oocyte. The level of FSH used here did not have a significant effect on the proportion of obviously degenerate oocytes in contrast to previous studies using small preantral bovine follicles (Nuttinck et al., 1996).

In contrast to cattle follicles, at the time of antrum formation in murine follicles, the oocyte is almost fully grown. The oocyte is known to regulate a variety of processes during follicular development (e.g. granulosa cell proliferation, steroidogenesis, cumulus cell expansion, for review see Buccione et al., 1990a). In murine follicles the comparatively more mature oocyte may play a different role in antrum formation perhaps in granulosa cell differentiation and/or sensitising the granulosa cells to FSH. In cattle, with a relatively immature oocyte, the involvement of the oocyte in antrum formation may be smaller perhaps relying more on the extrafollicular support from the surrounding tissue.

Although antrum formation is an important part of in vivo follicular architecture, growth of viable murine oocytes without antrum formation (Eppig and Schroeder 1989, Carroll et al., 1990) questions its necessity for in vitro oocyte production. It is possible that factors which may drive antrum formation in vivo, such as nutrient restriction and anoxia are different in vitro and that the follicle may survive without an antrum if the culture conditions were fulfilling the roles of the antrum.

During culture, localisation of granulosa cell proliferation to the layers nearest the oocyte was observed in all follicle classes. The localisation of proliferation effect was unlikely to be due to inhibition of proliferation in the outer layers of granulosa cells by factors in the medium as (i) high levels of theca proliferation were observed and (ii) peripherally positioned healthy oocytes showed proliferation localisation even if the surrounding granulosa cell layers were adjacent to the basement membrane. Localisation of granulosa cell proliferation was never seen in follicles where the oocyte was obviously degenerate. The relatively immature state of the oocytes studied here meant that oocyte viability could not be assessed directly. However, the relationship between granulosa

cell proliferation and oocyte morphology may indicate that the oocyte is still actively involved in follicular development at the end of culture.

Differentiation of granulosa cells may explain why proliferation is localised around the oocyte. Higher levels of proliferation are seen in cumulus cells, which surround the oocyte, compared to mural granulosa cells lining the follicle wall (Hirshfield and Midgley 1978). Differentiation of granulosa cells into these subpopulations occurs around antrum formation which coincides with the stages of follicles studied here. The observation that localisation only occurs when a morphologically normal oocyte is present may indicate a role of the oocyte in granulosa cell differentiation. Murine oocytes secrete a factor which stimulates granulosa cell proliferation (Vanderhyden et al., 1992) and the localisation of proliferation seen here may be the result of a similar bovine oocyte factor.

FSH reduced the percentage of follicles in the large preantral/early antral follicle class which exhibited localisation of granulosa cell proliferation. The reason for this is unclear but may depend on the specific state of differentiation of the granulosa cells or development of the oocyte as the effect was not observed in the other follicle classes examined.

Granulosa cells proliferated during culture. The estimated labelling index of the granulosa cells was not significantly affected by FSH. FSH has previously been shown to stimulate the proliferation of granulosa cells in culture (Rao et al., 1978). In mice, where an oocyte secreted factor seems to influence granulosa cell proliferation, FSH did not enhance the effect of the oocyte on proliferation (Vanderhyden et al., 1992). Our results support the existence of such a factor in bovine follicles. If this factor is maximally stimulating proliferation of granulosa cells at this stage of development, any effect of FSH may be difficult to detect and may explain why FSH did not stimulate granulosa cell proliferation in the bovine follicles cultured here, and smaller bovine follicles cultured by Nuttinck et al., (1996).

In conclusion, we have shown that bovine follicles can be isolated and grown in vitro where the effect of FSH on specific stages of follicle development could be examined. It is hoped that future studies will extend these studies to determine the roles of other hormones in follicular development and to investigate further the interactions between follicular somatic cells and the oocyte.

CHAPTER 7. APOPTOSIS IN ISOLATED AND CULTURED PREANTRAL AND EARLY ANTRAL FOLLICLES

7. 1. INTRODUCTION

Few follicles have the opportunity to develop to ovulation and most undergo atresia. Apoptosis is thought to be the mechanism by which follicles become atretic (Hughes and Gorospe 1991, Tilly et al., 1991). Apoptosis is a controlled physiological process by which cells are removed from tissues. The main feature which distinguishes apoptotic cell death from necrotic cells death is the regulated activation of Ca^{2+}/Mg^{2+} dependent endonucleases which cleave the internucleosomal linker DNA of the nucleus into multiples of 180-200 base pairs (reviewed by Schwartzman and Cidlowski, 1993). The regularly sized oligonucleosomes produced during apoptosis can be detected using end labeling and separation by agarose gel electrophoresis producing a characteristic laddering pattern (Wylie, 1980). In situ hybridization, which can detect apoptosis in individual cells whilst preserving follicular architecture (Palumbo and Yeh, 1994), has shown that granulosa cells are the main cell type undergoing apoptosis in atretic follicles (Chun et al., 1996). In contrast, necrosis is characterised by rupture of organelles and release of proteolytic enzymes which digest histones, allowing random digestion of DNA by lysosomal deoxyribonuclease (review: Schwartzman and Cidlowski, 1993).

Atresia of follicles occurs at all stages of development (Hsueh et al., 1994). The fate of a follicle is modulated by factors in its surrounding environment and survival and atretogenic factors have been identified. Gonadotrophins, growth factors (EGF, TGFα, bFGF, IGF-I) and certain steroids (oestrogens) suppress apoptosis whilst other

factors (androgens, Gn-RH) induce apoptosis (Tilly et al., 1992, Billig et al., 1993, 1994, Chun et al., 1994). It is likely that the effects of these factors on apoptosis in follicles depends on the precise stage of development studied. Previous studies have concentrated on apoptosis in large preovulatory follicles. It is not known whether apoptosis is the mechanism underlying atresia of smaller follicles. In this study a system for the isolation and culture of intact bovine follicles will be used to examine apoptotic cell death in follicles as they progress from the large preantral to the early antral stages of follicular development.

7.2 METHODS

7.2.1. Follicle Isolation

Follicles were isolated as described previously (chapter 2). Isolated follicles were divided into 3 size classes: preantral (approximately 90 μm to 150 μm), large preantral/early antral (approximately 150 μm to 210 μm) and antral (approximately 210 μm to 270 μm).

7.2.2. Follicle storage

At the end of the dissection period, follicles from each group were either snap frozen immediately or cultured for 24 hours before snap freezing. Snap freezing of the follicles was achieved by placing them in micro tubes (cap open) with a minimal amount of medium and dipping the end of the tube in liquid nitrogen. Follicles were then stored at -20 °C.

7.2.3. Follicle Culture

4 well tissue culture plates were prepared as described in chapter 3. 1 ml of serum free culture medium (TCM199 (Cat. no. M0148, Sigma, Poole, Dorset), 3 mg/ml BSA (bovine fraction V, Sigma), 50 μg/ml gentamycin (Life Technologies, Paisley, UK), 250 mg/ml sodium pyruvate (Sigma), 50 μg/ml insulin (Sigma), 10 μg/ml transferrin (Sigma) was used to wash the collagen before fresh medium was added. Culture wells were incubated at 39 °C, 5 % CO_2 in a humidified incubator for at least one hour before the follicles were added. Four follicles were added to each culture well.

7.2.4. Low molecular weight DNA extraction

Groups of between 60 and 90 follicles were pooled into one tube for DNA extraction. More follicles were used for the preantral follicle groups which were

smaller and therefore contained less DNA than the larger early antral follicles. DNA was also extracted from the control samples: (i) thymocytes treated with dexamethosone (Sigma) for 24 hours and (ii) bovine ovarian stromal tissue. 200 µl of homogenisation buffer (0.1 M NaCl, 0.01 M EDTA (pH 8), 0.3 M Tris HCl (pH 8), 0.2 M sucrose), 12.5 µl 10% SDS and 0.06 µg proteinase K (Sigma) were added to each tube, mixed briefly and allowed to incubate at 65 ^{o}C overnight. Once the tissue was fully dissolved the tubes were spun at 5000g for 20 minutes at 4^{o}C. The supernatant was collected and placed in a polypropylene micro tube (Bio-rad, Hercules, Ca, USA) and an equal quantity of cold (4 ^{o}C) phenol:chloroform:isoamyl alcohol mix (25:24:1, pH 7.2) was added, mixed for 30 seconds and spun for 1 minute at 10,000 g. The upper phase was then removed to a fresh micro tube and an equal volume of cold (4^{o}C) chloroform:isoamylalcohol mix was added and mixed and spun as before. The upper phase was removed and placed in a fresh micro tube and ice cold ethanol was added (2.5x volume of sample). The tube was stored at -70 ^{o}C overnight to allow the DNA to precipitate.

The samples were spun at 14000 g for 30 minutes at (4^{o}C) and the alcohol removed. 200 µl of ice cold 80 % alcohol was added to the sample and respun as before. The alcohol was removed and the pellet allowed to air dry. Once dry, the pellet was resuspended in 23 µl of TE buffer (pH 8). Three microliters of the sample was suspended in distilled water and its absorbance read at 260nm and 280nm using a spectrophotometer (Beckman DU-65, address). The absorbance at 260nm was used to determine the quantity of nucleic acid in the sample. The ratio of the absorbances (260 nm/280 nm) is used to calculate the purity of the sample; a pure DNA sample has a ratio of absorbances of 1.8 (Sambrook et al., 1989), lower ratios are caused by protein or phenol contamination making calculation of the quantity of DNA difficult. The remainder of the sample was stored at -20 ^{o}C until required.

7.2.5. 3'end labelling of DNA

All procedures using ^{32}P labelled dd ATP were conducted behind lead loaded perspex, with double gloves and radiation monitoring in accordance with local rules. To 2 µg of the DNA sample to be labelled (or 0.2 µg of the DNA ladder (DNA size standard (low range), Bio-Rad Laboratories, Richmond, Ca, USA)) 10 µl of 5X reaction buffer (1 M sodium cacodylate, 0.125 M tris HCl, 1.25mg/ml BSA, pH 6.6), 5 µl 10 mM $CoCl_2$, 3µl [α-^{32}P]ddATP (50 mCi) (Amersham,Buckinghamshire, UK)(1 µl for ladder) and 1 µl of terminal transferase (calf thymus) (0.5 µl for ladder) (Sigma) were added and incubated at 37 ^{o}C for 60 minutes. The reaction was stopped by the addition of 5 µl of 0.25 M EDTA.

7.2.6. Separation of labelled DNA from free ^{32}P ddATP

Sephadex spin columns (either prepared in the lab (Sambrook et al., 1989) or preprepared (Quick spin columns, cat. no. 1273 965, Boehringer Mannheim, Sussex, U.K.)) were used to remove unincorporated ddATP from the labelled DNA. The preprepared columns were spun once (3min, 1500 g), washed with PBS and respun for 3 min 1500 g. The DNA sample was added to the top of the column and spun for 3 minutes at 1500 g and the purified radiolabelled DNA was eluted into the collection tube.

7.2.7. Separation of DNA by agarose gel electrophoresis

The gel was prepared by heating a 1.5 % solution of agarose (ultrapure, electrophoresis grade, Life Technologies, Paisley, U.K.) in TAE 1X buffer until fully dissolved. The gel was allowed to cool slighty to around 60 ^{o}C before pouring into the gel bed. One of two gel electrophoresis systems were used depending on the number of samples to be analysed: a minigel system was used for a small number of samples (Origo, model H-2, Anachem, Luton, UK) and a 12 well system used for

larger sample numbers (Hybaid, Middlesex, U.K.). The wells of the electrophoresis chamber were filled with 1X TAE buffer so that the the gel was slightly covered. The volume of sample in the collection tube was measured by pippetting and tracking dye was added to the sample at 1/10 volume of the sample. The DNA samples were loaded into the wells of the gel, leaving an empty well between the controls or ladder and the samples to be examined. The gel was run at 80 V until the tracking dye had moved two thirds olong the length of the gel. The electrophoresis system was then drained and the gel lightly blotted with filter paper, wrapped in cling film and placed on a bed of filter paper in the gel dryer. The gel was allowed to dry in a vacuum (without heat) until wafer thin.

7.2.8. Gel Exposure

After drying, the gel was wrapped in a second layer of cling film and placed in a developing box and overlain with x-ray film (X-OMAT AR, Eastman Kodak Co, Rochester, NY, USA). The gel was exposed to the film at -80 ^{o}C for 1 hour initially. The x-ray film was developed and used to give an indication of the best exposure time. A phosphoimager (Molecular Dynamics, Buckinghamshire, U.K.) was used if difficulty was experienced resolving lanes due to different levels of radioactivity between samples on the same gel. This method allowed changes in radioactivity along each lane to be analysed using an image analysis system (Image Quant, Molecular Dynamics) without multiple film exposures to determine whether DNA laddering was present.

7.3. RESULTS

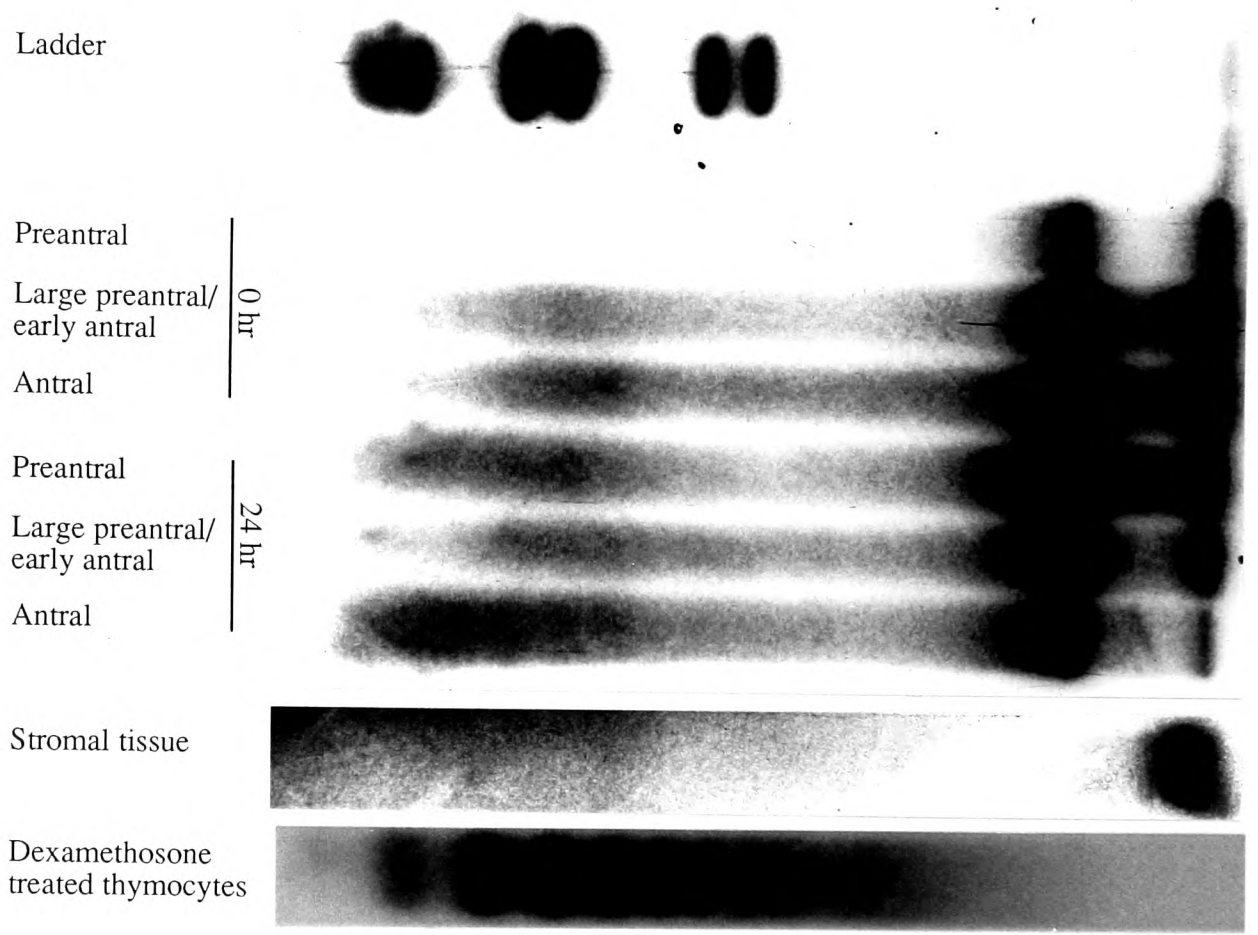
Analysis of total cellular DNA from isolated bovine follicles by 3' end labelling indicated that internucleosomal fragmentation of the DNA into multiples of 180-200 base pairs had not occurred to a significant degree in any of the follicle classes studied either before or after culture for 24 hours (figure 7.1). The positive control, DNA isolated from dexamethosome treated thymocytes, did show the characteristic laddering pattern indicative of apoptotic DNA fragmentation (figure 7.1). The laddering pattern was detected both on the x-ray film and the phosphoimager (figure 7.2) when exposed to the gel.

The phosphoimager allowed an image of the gel to be analysed by image analysis. Analysis of the changes in pixel density along each lane allowed sensitive measurement of the changes in radioactivity. The laddering pattern normally seen when apoptosis had occurred was shown as a series of peaks and troughs on a radioactivity (pixel counts) against migration distance along the gel plot (figure 7.2) whereas a relatively constant level of radioactivity was seen when apoptosis does not occur (figure 7.2). Results gained using the phosphoimager system agreed with those obtained using the x-ray film.

Due to the procedure used to isolate the follicles, some stromal tissue was isolated with the follicles. Stromal tissue was included as a control to exclude the possibility that its DNA may be contribute to any apoptotic DNA fragmentation observed. Stromal tissue DNA control did not show detectable levels of apoptosis (figure 7.1).

A large number of follicles were required for sufficient quantities of DNA to be isolated for analysis. In addition, some difficulty was experienced in redissolving the extracted DNA which may account for the slightly lower quantity of DNA recovered than expected. However, the experiment was repeated three times and no detectable differences were seen between repetitions.

Figure 7.1. Autoradiograph showing DNA separated by agarose gel electrophoresis. DNA was isolated from preantral, large preantral/early antral and antral bovine ovarian follicles either immediately after isolation (0 hr) or after culture for 24 hours (24hr). No significant levels of DNA fragmentation by apoptosis into multiples of 180-200 base pairs (shown by ladder) was observed.


Ladder
Preantral
Large preantral/
early antral
Antral
0 hr
Preantral
Large preantral/
early antral
Antral
24 hr
Stromal tissue
Dexamethosone
treated thymocytes

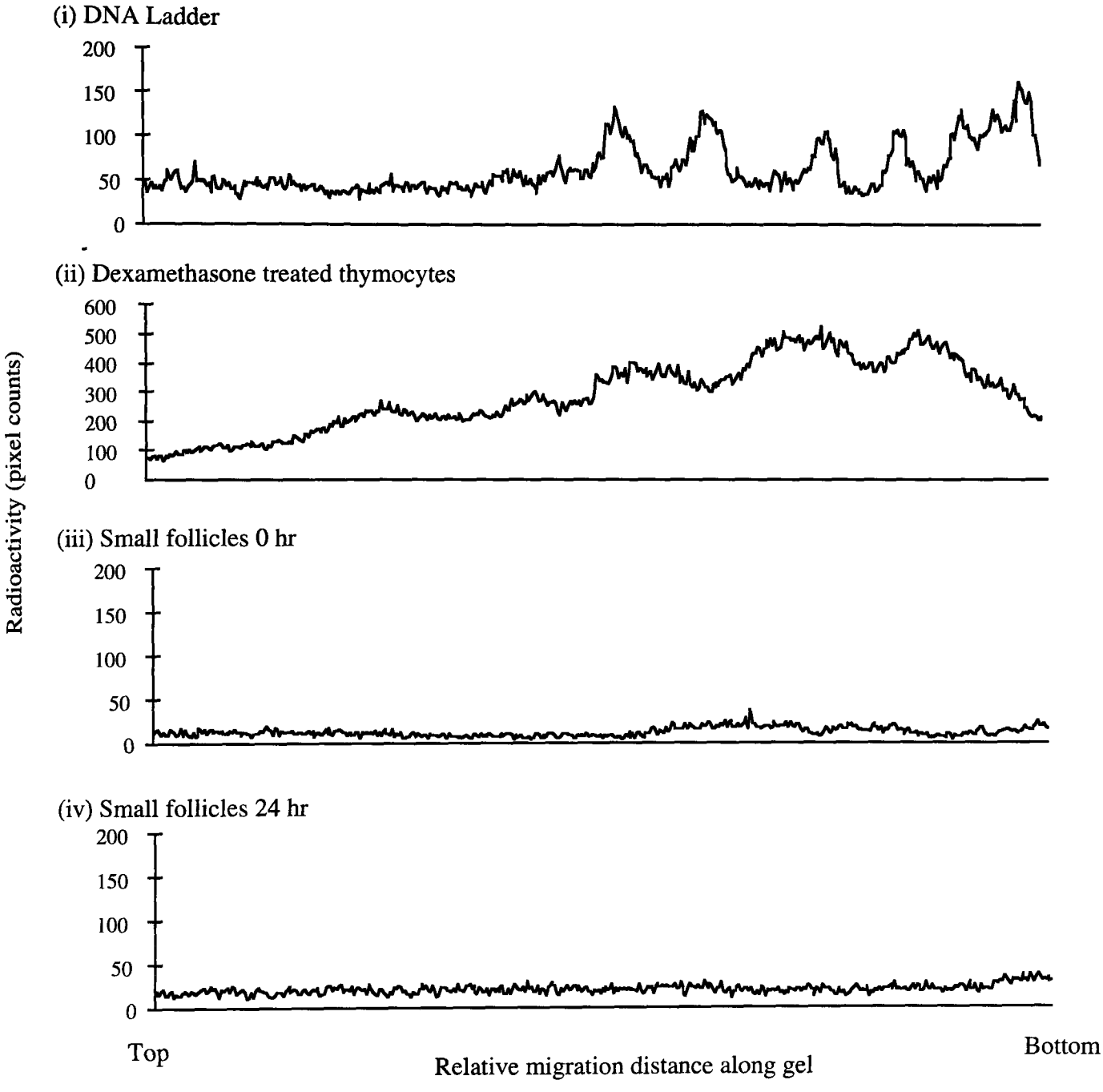


Figure 7.2. Counts against distance along the gel plots showing the separation of DNA by agarose gel electrophoresis. The gels were exposed and developed using a phosphoimager system. DNA fragmentation was seen as a peaks and trough effect in the ladder and dexamethosome treated thymocytes. No detectable DNA fragmentation by apoptosis was observed in follicles at isolation or following culture for 24 hours.

7.4. DISCUSSION

Apoptosis is thought to be the mechanism by which all ovarian follicles become atretic (Hughes and Gorospe, 1991, Tilly et al 1991, Hsueh et al., 1994). Studies of isolated granulosa cells or large antral follicles have shown that apoptosis is detectable before any morphological signs of atresia (Hughes and Gorospe, 1991, Jolly et al., 1994) and is only present in atretic follicles (Tilly et al., 1991). It is not known whether apoptosis is the mechanism of atresia in smaller follicles. The aims of this study were therefore: 1. to examine the presence of apoptosis in morphologically normal bovine follicles from the preantral to antral stage of follicular development and 2. to determine whether apoptosis can be induced in these follicles using conditions which result in apoptosis of granulosa cells in large antral follicles.

The precise sequence of events which lead to apoptosis and follicular atresia are unknown, although a large number of factors have been implicated in the regulation of apoptosis in the ovary (reviews; Schwartzman and Cidlowski, 1993, Hsueh et al., 1994). The effects of these factors on apoptosis have been examined almost exclusively in large antral follicles and mixed granulosa cell populations. Recently it has become clear that the effect of these factors may depend on the stage of follicle studied (Billig et al., 1994, Luciano et al., 1994, Chun et al., 1996, Manabe et al., 1996). Although the largest proportion of atretic follicles in the ovary are at the early antral stage of development (Hirshfield and Midgley, 1978) there have been few studies of apoptosis in this follicle population. The present study is the most detailed analysis to date of apoptosis in preantral and early antral bovine ovarian follicles.

The only previous studies of apoptosis in preantral and early antral follicles used <u>in situ</u> 3' end labelling (Palumbo and Yeh, 1994, Billig et al., 1994). However, other forms of DNA damage, including necrosis, also result in labelling using this method. Few studies have confirmed <u>in situ</u> analysis of apoptosis in preantral and early antral follicles by separation of labelled DNA by agarose gel electrophoresis due

to difficulty of obtaining sufficient quantities of DNA. Three' end labelling of DNA and separation by agarose gel electrophoresis is 100 times more sensitive than the ethidium bromide staining techniques used in initial studies of apoptosis and has allowed smaller quantities of DNA to be used (Tilly et al., 1991). Despite our use of the 3'end labelling of isolated DNA technique, large numbers of follicles were still required for this study due to the small size of the follicles.

Granulosa cells are involved in the regulation of oocyte growth (review: Buccione et al., 1990b). The role of the oocyte in follicular cell proliferation and function is also increasingly being recognised as an important component of normal follicular development. The oocyte produces factors which stimulate granulosa cell steroidogenesis (Vanderhyden et al., 1993), proliferation (Vanderhyden et al., 1992) and cumulus cell expansion (Buccione et al., 1990b, Salustri et al., 1990a). The study of apoptosis using cultures of isolated granulosa cells, in the absence of the modulating influence of the oocyte, may not be truly representative of the events occurring in the ovary and a recent study has indicated that the oocyte may play a regulatory role in the induction of granulosa cell apoptosis (Hakuno et al., 1996). However, in those studies (Hakuno et al., 1996), the normal communication between the oocyte and granulosa cells was disrupted. The system we have developed for the isolation and culture of intact bovine ovarian follicles where oocyte-somatic cell communication is preserved presents a more physiological model in which to examine apoptosis at a precise stage of follicular development.

In this study, the presence of apoptosis in bovine ovarian follicles over the size range where antrum formation occurs (Monniaux et al., 1984) was not detected in the morphologically normal follicles selected at isolation. The lack of apoptosis in the follicles at isolation makes them suitable for examining causes of the onset of apoptosis and a good starting material for in vitro follicle growth studies. In contrast to this work, evidence of apoptosis has been found in apparently healthy preovulatory follicles before any signs of atresia (Jolly et al., 1994, Hughes and Gorospe, 1991).

The differentiated state of the granulosa cells in the follicles studied here compared to preovulatory follicles examined in previous studies (Jolly et al., 1994, reviewed by Hsueh et al., 1994) may account for the absence of apoptosis. In large antral or preovulatory follicles granulosa cells are differentiated into cumulus cells which have a closer association with the oocyte and are more mitotically active than mural granulosa cells which are larger and more steroidogenically active (Lederer et al., 1995, Armstrong et al., 1996). Apoptosis has been detected in mural granulosa cells but not in cumulus granulosa cells of atretic follicles (Luciano et al., 1994, Manabe et al., 1996). Whether cumulus cells are less likely to undergo apoptosis because they are proliferating has still to be shown as the nature of the relationship between mitosis and apoptosis has yet to be defined (Earnshaw, 1995). Alternatively, closer association of the cumulus cells with the oocyte than the peripherally positioned mural granulosa cells may account for the differences in apoptosis. Whether high levels of proliferation of granulosa cells or a more intimate association with the oocyte in preantral follicles are possible reasons for the lack of apoptosis in preantral follicles has yet to be shown.

The oocyte is important in the regulation of follicular development and may be involved in suppression or induction of follicular atresia. In preantral follicles, oocyte degeneration often proceeds atresia of the rest of the follicle (Hakuno et al., 1996) indicating a possible regulatory role of the oocyte in follicular atresia. Coculture of oocytes with granulosa cells has been shown to increase the percentage of apoptotic granulosa cells following culture (Hakuno et al., 1996). The Fas ligand, known to be involved in the induction of apoptosis through the interaction with Fas on the target cell surface (Quirk et al., 1995), has been shown to be produced by oocyte (Hakuno et al., 1996). However, Fas has not yet been observed in the early stages of follicular development (Hakuno et al., 1996) as studied here and it is possible that another

mechanism mediating apoptosis may exist. This further supports the idea that atresia in smaller follicles is different from that of large antral and preovulatory follicles.

Culture of granulosa cells from healthy large antral follicles for 24 hours in serum and gonadotrophin free medium results in the spontaneous onset of apoptosis (Tilly et al., 1992, Luciano et al., 1994). Follicles isolated here did not show any signs of apoptosis following culture for 24 hours in these conditions. It is likely that the earlier stages of follicles examined here compared to previous studies (Tilly et al., 1992, Luciano et al., 1994) could account for the differences in results. However, recent work has shown the presence of apoptosis following culture of early antral rat follicles in serum free medium (Chun et al., 1996). This indicates that the spontaneous onset of apoptosis during serum free culture may depend on both the stage of follicles examined and the species.

An alternative mechanism of programmed cell death which is not apoptotic may be involved in follicular atresia (Luciano et al., 1994). This may account for the lack of apoptosis in the follicles studied here, in atretic rat follicles (Palumbo and Yeh, 1994) and degenerate oocytes (Hakuno et al., 1996). However, it is also likely that in preantral and early antral follicles, gonadotrophins are less potent survival factors and that culture of these follicles in their absence had little effect on induction of apoptosis.

Most follicles within the ovary undergo atresia. Apoptosis is the underlying mechanism of atresia at the penultimate stages of follicular development. Whether cell death is occurring by a mechanism other than apoptosis at earlier stages of follicular development is an aim for future research.

CHAPTER 8: THE ROLE OF THE BOVINE OOCYTE IN CUMULUS CELL EXPANSION

8.1. INTRODUCTION

The oocyte and the companion somatic cells comprising the follicular unit maintain a close association throughout development from primordial to preovulatory stages. The inter-communication between the oocyte and the somatic cells is recognised as an important component in growth and differentiation (Salustri et al., 1990a, b, Buccione et al., 1990b, Eppig et al., 1993 a, b, Vanderhyden et al., 1992, for reviews see Buccione et al., 1990a, Eppig, 1991). In the mouse this communication has been shown to be important in cumulus cell expansion.

Cumulus cell expansion occurs around the time of ovulation (precise timing is species dependant, Motlik et al., 1986), where, in response to the gonadotrophin surge, the cumulus cells surrounding the oocyte secrete hyaluronic acid forming a gelatinous matrix around the oocyte (Dekel and Kraicer, 1978). The gelatinous matrix is thought to be important in pick up of the complex by the oviductal fimbrae and also in fertilisation (Meizel, 1985, Salustri et al., 1989). Moreover, expansion disrupts gap-junctional communication between the cumulus cells and the oocyte (Motlik et al., 1986) and has been implicated in the control of meiotic arrest (Eppig and Downs 1984, Downs, 1993). Cumulus cell expansion of isolated intact cumulus oocyte complexes (COC) can be stimulated in vitro and the mature complex used for in vitro fertilisation (Madison et al., 1992). It is easily recognised in vitro, where the arrangement of cumulus cells changes from a tightly compact configuration around the oocyte to a larger glistening mass, within which individual isolated cumulus cells can be seen (Eppig, 1980a).

The oocyte has been shown to play an important role in cumulus cell expansion in the mouse and rat (Buccione et al., 1990b, Salustri et al., 1990a, b, Vanderhyden, 1993). In response to FSH and in the presence of serum, murine cumulus cells undergo hyaluronic acid synthesis and expansion, only if fully grown, meiotically competent oocytes or media conditioned by these oocytes is present (Buccione et al., 1990b, Salustri et al., 1990a, b). Therefore an oocyte or oocyte secreted factor(s) is essential for murine cumulus cell expansion (Buccione et al., 1990b, Salustri et al., 1990a). Conditioning media with other cell types (granulosa cells, sertoli cells, spermatozoa or fibroblasts) has failed to enable cumulus cell expansion (Buccione et al., 1990b). The factor has been termed the cumulus expansion enabling factor (Buccione et al., 1990b) and is secreted specifically by the oocyte (Buccione et al., 1990b) in murine species at least. In the pig, although such a factor is secreted by the oocyte, it does not appear to be essential for expansion (Prochazka et al., 1991, Vanderhyden, 1993, Singh et al., 1993) . It is not known whether bovine oocytes secrete a cumulus expansion enabling factor, if such a factor is required for expansion or if it is produced specifically by the oocyte.

Simple characterisation of the murine cumulus expansion enabling factor was carried out by Eppig et al., 1993a. So far it is known that it has a nominal molecular weight of 100-300kd and is likely to be a protein or dependant on a protein for its activity. Further analysis of the factor has been complicated by: 1. Collection of large quantities of the factor is limited by the small number of fully grown oocytes obtained (Eppig et al., 1993a). 2. The factor is unstable, losing its activity after several hours (Eppig et al., 1993a). A method of collection and storage of the factor, maintaining activity would help in its elucidation.

The aims of this study were therefore: 1. To determine whether bovine cumulus cells require the presence of an oocyte or oocyte secreted factor for cumulus cell expansion to occur. 2. to establish if bovine oocytes produce a factor which enables cumulus cell expansion in the mouse. 3. to devise a simple method for the

storage of bovine oocyte cumulus expansion enabling factor. 4. To determine whether other cells within the follicle produce the cumulus expansion enabling factor.

8.2. MATERIALS AND METHODS

8.2.1. Requirement of the bovine oocyte for cumulus cell expansion

8.2.1.1. Isolation of cumulus oocyte complexes (COC).

Bovine ovaries were obtained from an abbattoir at 30°C, washed with industrial methylated spirits and placed in PBS (Unipath Ltd., Basingstoke, U.K.) with 50 mg/l Gentamycin (Sigma Chemical Co., Poole, Dorset, U.K.). 2-6mm follicles were aspirated using a 25G needle and syringe. The follicular fluid collected was allowed to settle for 15 minutes and the overlying fluid drawn off. The residue was resuspended in dissection medium (M199 (cat. no. 21180-013 GIBCO-BRL, Life Technologies, Paisley U.K.), 0.03M Hepes (Sigma, Poole, Dorset, U.K.), 0.13mM Kanomysin Monosulphate (Sigma, Poole, Dorset, U.K.), 10% foetal calf serum (Globepharm Ltd., Surrey, U.K.), and 50μM IBMX (Sigma, Poole, Dorset, U.K.)) and good quality COC complexes (fully cumulus enclosed with evenly pigmented oocyte cytoplasm) were selected under the dissecting microscope.

8.2.1.2. Microsurgical Oocyte Content Removal (Oocytectomy)

Microsurgical removal of the contents of the oocyte was performed as described previously. (Buccione et al., 1990b, Prochazka et al., 1991). Briefly, using micromanipulators with a holding pipette (20μm diameter hole) and a fine glass needle (figure 8.1a) the complex is held under negative pressure (figure 8.1b) and the oocyte pierced through both sides of the zona pellucida (figure 8.1c). The contents of the oocyte are then aspirated through the holding pipette on removal of the glass needle, resulting in temporary deformation of the zona pellucida (figure 8.1d). Almost all of the oocyte contents are removed in this way leaving an essentially intact complex without the oocyte - the oocytectomised complex (OOX) (figures

8.1e, 8.2). Occasionally this procedure resulted in loss of the cumulus cells and only OOX complexes with at least 3 complete cumulus cell layers were used in this study.

8.2.1.3. Measurement of cumulus cell expansion

Intact and OOX complexes were rinsed twice in the culture medium (M199 (GIBCO-BRL, Life Technologies, Paisley U.K.), 0.02M Hepes (Sigma, Poole, Dorset, U.K.), 0.026M sodium bicarbonate (Sigma, Poole, Dorset, U.K.), 10% foetal calf serum (Globepharm Ltd., Surrey, U.K.) and 6mIU FSH (Pergonal, Serono Laboratories U.K. Ltd, Welwyn Garden City, U.K.) to remove any IBMX. Groups of COC or OOX complexes were added to 50µl drops of culture medium under paraffin oil and incubated for 24 hours to allow maximal expansion at 39°C and 5% CO_2. A sample of COC were cultured in the absence of FSH to determine the level of spontaneous expansion. In addition, a group of OOX complexes were preincubated for 2 hours before culture.

At 24-26 hours, the degree of expansion was scored using a qualitative scale (table 8.1) and the results analysed using a χ^2 test.

8.2.2. Role of the zona pellucida, oocyte membrane or residual oocyte cytoplasm in bovine cumulus cell expansion

It is possible that the zona pellucida, oocyte membrane or residual oocyte cytoplasm may provide the bovine OOX complex with the correct stimulus for cumulus cell expansion to occur. To examine this possibility, groups of 10 clumps of cumulus cells (without any oocyte component or zona pellucida) were microdissected from COC and incubated at 39°C, 5% CO_2 in 50µl drops of culture medium under paraffin oil for 24-26hours. Expansion was then scored as before.

8.2.3. Production of a factor which enables murine cumulus cell expansion

Murine OOX complexes are known to require the presence of an oocyte secreted factor before cumulus cell expansion can occur (Buccione et al., 1990b, Salustri et al., 1990a). In this experiment the production of such a factor by bovine oocytes was analysed.

8.2.3.1. Isolation and preparation of intact and OOX murine complexes and denuded bovine oocytes.

Ovaries were removed from 35 day old, 48hr eCG primed C57black6/CBA F1 mice and placed in M2 medium (Hogan et al., 1986) with 50μM IBMX. Large follicles were punctured under the dissecting microscope using 25G needles and murine COC released. Oocytectomy was performed as before using murine COC in 200μl drops of M2 medium and IBMX under paraffin oil.

Bovine COC were isolated as before. Denuded bovine oocytes were prepared by repeated pipetting using a 1ml pipette to remove almost all the cumulus cells (Eppig and Downs, 1984, Vanderhyden et al., 1993).

8.2.3.2. Assessment of cumulus cell expansion

After rinsing twice in culture medium to remove the IBMX, groups of either 9-15 mouse COC, mouse OOX or mouse OOX plus 50 denuded bovine oocytes were added to 50μl drops of culture medium under paraffin oil. The micro-drops were cultured for 24-26hours and examined for cumulus cell expansion. Results were analysed using the Student's t-test.

8.2.4. Examination of the secretion of the bovine cumulus expansion enabling factor

The aim of this experiment was to determine if the beneficial effect of the bovine oocytes is mediated by direct contact of the bovine oocytes with the murine OOX or by a secreted factor.

Denuded bovine oocytes were prepared as before. Culture medium was conditioned by adding denuded bovine oocytes at a concentration of 1 oocyte/μl and incubating for 4 hours at 39°C, 5% CO_2. After this time, the conditioned medium was collected and used to prepare 50μl drops under paraffin oil. Nine-15 murine OOX were added to each drop and expansion examined after 24-26 hours. The results were analysed using the Student's t-test.

8.2.5. Storage of conditioned medium

To determine whether the medium conditioned by bovine oocytes could be stored frozen whilst maintaining its cumulus expansion enabling activity, aliquots of conditioned medium (prepared as above) were frozen (-20°C) immediately after collection for up to a month. As required, aliquots were heated to 39°C and divided into 50ul drops. Nine-15 murine OOX were added to each drop and examined for expansion at 24-26 hours and the results analysed using the Student's t-test.

8.2.6. Production of cumulus expansion enabling factor by mural granulosa cells

The cumulus expansion enabling factor may be produced by other somatic cells within the bovine follicle. To examine this possibility, the effect of mural granulosa cells on cumulus cell expansion was determined.

8.2.6.1. Isolated mural granulosa cells

Bovine follicles (2-6mm diameter) were dissected from the ovaries using a scalpel. The follicles were sliced open and rinsed in dissection medium. In fresh medium, the insides of the follicle wall were scraped with the scalpel. The cell suspension was then centrifuged at 225 g for 15 minutes. The supernatant was withdrawn, fresh medium added and the tube tipped several times to wash the cells. The cells were spun as before and the supernatant withdrawn. The top layer of cells was resuspended in 0.5ml of culture medium by gentle pipetting and then transferred to a tube containing 1.5ml of culture medium. The cell concentration was adjusted to 3-4 x 10^6 cells/ml by addition of fresh culture medium. The cell suspension was used to make 50µl drops under paraffin oil.

8.2.6.2. Mural granulosa cells attached to the follicle wall

Bovine follicles (2-6mm) were isolated and prepared as described above. Small sections of the follicle wall and adherent mural granulosa cells ($2mm^2$) were dissected using a scalpel and added to 50µl drops of culture medium.

8.2.6.3. Assessment of cumulus cell expansion.

Between 8 and 20 murine OOX were added to each drop and cultured for 24-26 hours. Groups of intact murine COC or murine OOX alone were cultured as controls. The degree of expansion was scored as before. Results were analysed using a Student's t-test.

a.

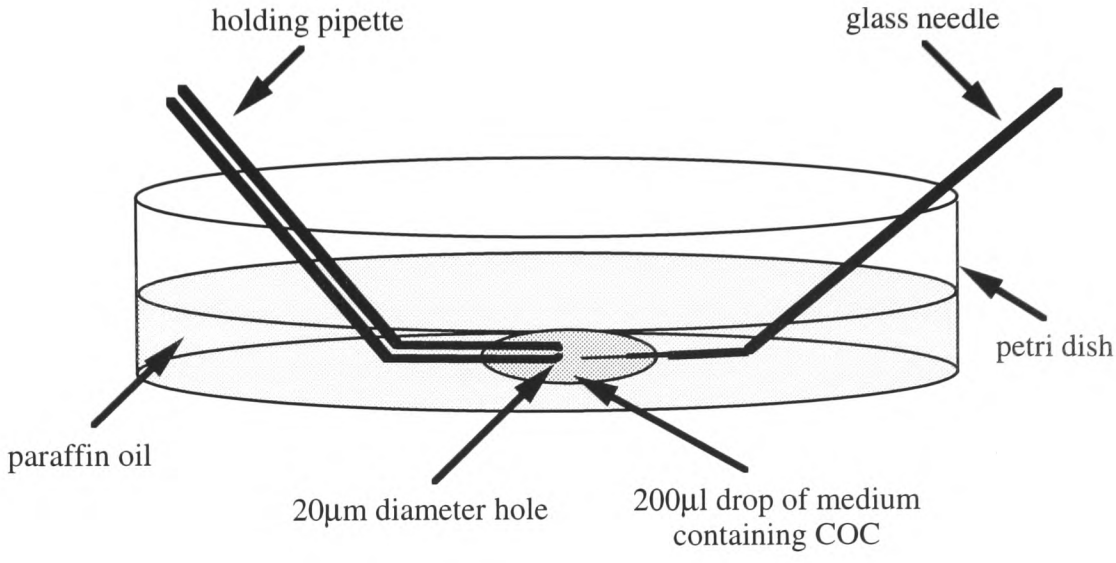
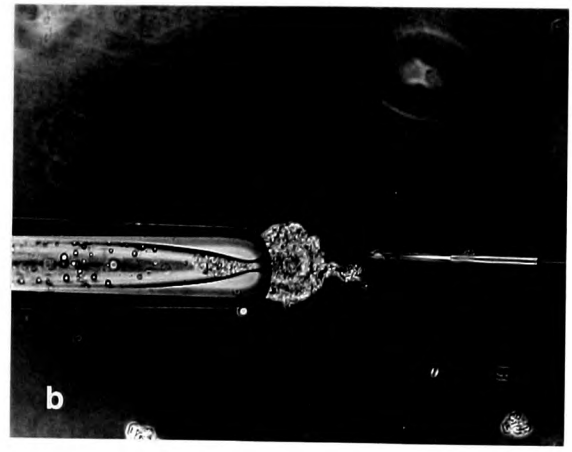


Figure 8.1. Microsurgical oocyte content removal (oocytectomy): a. a holding pipette and glass needle are connected to micromanipulators under an inverted microscope. Micro-drops of medium under paraffin oil in a petri dish contain the cumulus oocyte complexes (COC) to be oocytectomised b. the COC is held under negative pressure using the holding pipette c. the glass needle is used to pierce the oocyte d. on withdrawl of the glass needle, the oocyte contents are aspirated through the holding pipette, resulting in temporary deformation of the zona pellucida e. oocytectomised complexes.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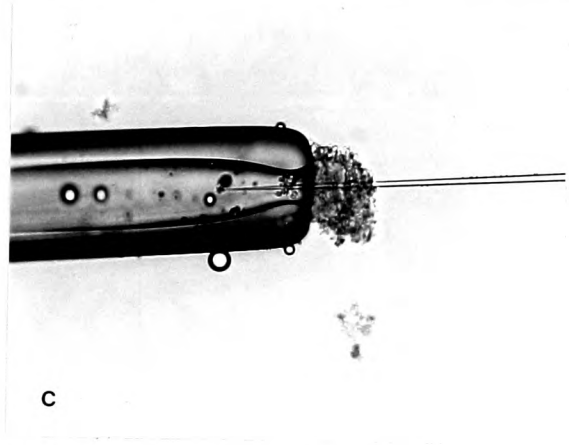
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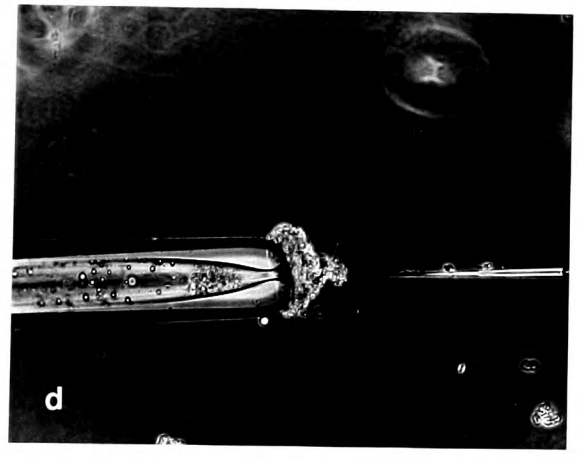
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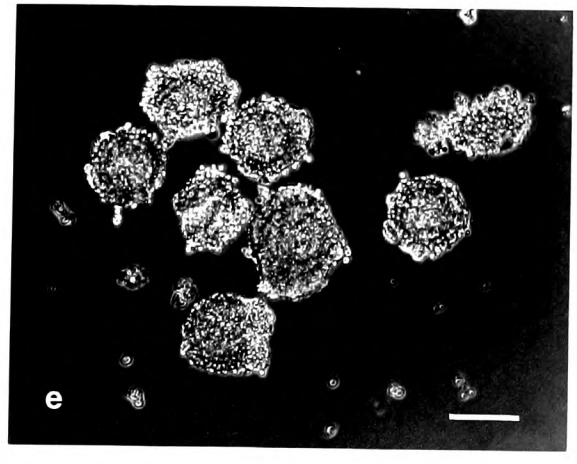

e

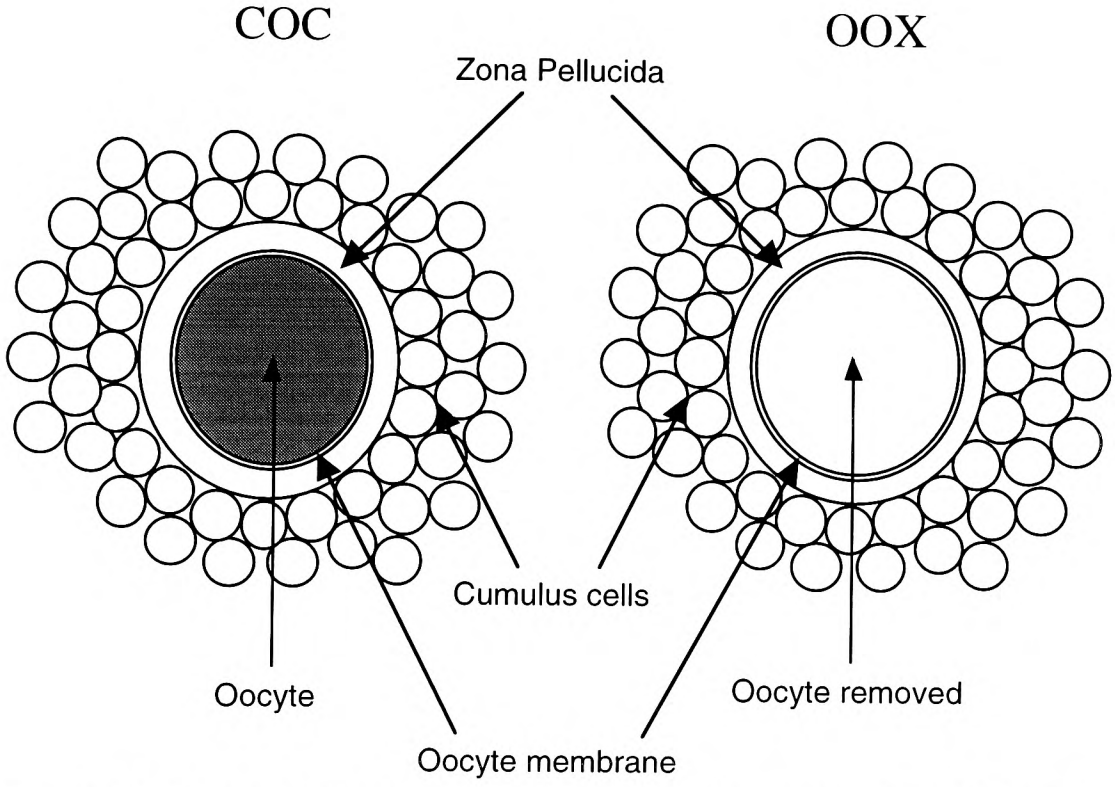


Figure 8.2 Schematic diagram showing cumulus oocyte complex (COC) and oocytectomised complex (OOX). Oocytectomy removes the oocyte contents leaving the oocyte membrane, zona pellucida and arrangement of cumulus cells intact

1	2	3	4
No expansion	Minimal expansion; slight glistening of complex	Expansion; all layers of cumulus cells expand except the corona radiata	Maximum expansion; all layers of cumulus cells expand

Table 8.1. Table showing scale used to measure the degree of cumulus cell expansion

8.3. RESULTS

8.3.1. Requirement of the bovine oocyte for cumulus cell expansion

Cumulus cell expansion (groups 3 and 4 in the expansion scale, table.1) was observed in both the intact COC and OOX complexes (table 8.2, $p>0.05$, figure 8.4). The presence of bovine oocytes during cumulus cell expansion does not therefore appear to be necessary in cattle. Preincubation of bovine OOX for 2 hours before culture did not prevent cumulus cell expansion. Removal of FSH resulted in fewer complexes undergoing cumulus cell expansion although some spontaneous expansion was observed,

8.3.2. Role of the zona pellucida, oocyte membrane or oocyte cytoplasm in cumulus cell expansion

Culturing clumps of bovine cumulus cells for 24-26 hours with or without bovine oocytes had no effect on their ability to undergo cumulus cell expansion (table 8.3). This suggests that during bovine cumulus cell expansion, the presence of the oocyte, structural components of the oocyte or zona pellucida is not required.

8.3.3. Production of a factor which enables murine cumulus cell expansion

Removal of murine oocyte contents prevents cumulus cell expansion of the complexes following culture (figure 8.3, $p<0.05$, figures 8.5 c and d) as has been shown previously (Buccione et al., 1990b). The presence of bovine oocytes in culture permits cumulus cell expansion of murine OOX complexes as shown in figures 8.3 and 8.5e.

8.3.4. Examination of the secretion of a bovine produced cumulus expansion enabling factor

Bovine oocyte conditioned medium allowed cumulus cell expansion in murine OOX complexes (figure 8.3, $p>0.05$). As no direct contact between bovine oocytes and the murine OOX complexes was required to stimulate expansion this suggests that bovine oocytes condition the culture medium by secreting a factor which enables expansion to occur.

8.3.5. Storage of conditioned medium

Frozen/thawed bovine oocyte conditioned medium still permits murine OOX complexes to undergo cumulus cell expansion (see figure 8.3, $p>0.05$). The cumulus expansion enabling factor secreted by bovine oocytes can therefore be stored frozen, maintaining its expansion promoting ability for a period of up to at least one month. This may allow stocks of the medium to be collected and stored for further analysis.

8.3.6. Production of cumulus expansion enabling factor by mural granulosa cells

Coculture of murine OOX with bovine mural granulosa cells as either isolated cells or when attached to the follicle wall had no effect on their ability to undergo cumulus cell expansion (figure 8.6, $p>0.05$). This indicates that the bovine cumulus cell expansion enabling factor is not produced by the mural granulosa cells of the follicle.

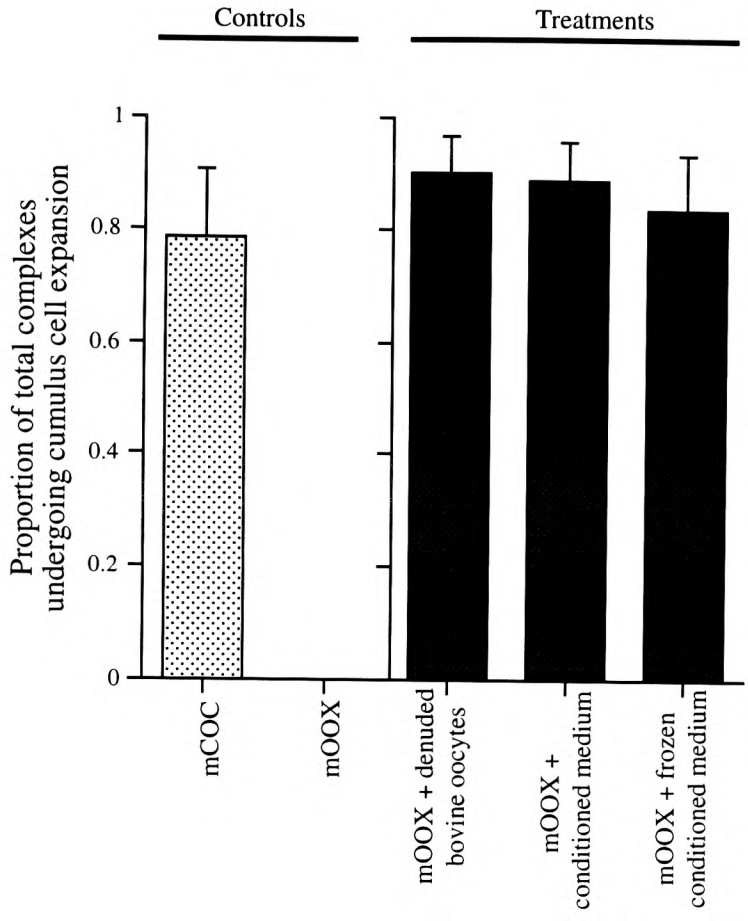


Figure 8.3. Graph showing proportion of expanded murine cumulus oocyte complexes (mCOC), (n=126, 8 repeats), murine oocytectomised complexes (mOOX) (n=49, 5 repeats), mOOX + denuded bovine oocytes (n=56, 6 repeats), mOOX + bovine oocyte conditioned medium (n=59, 6 repeats) and mOOX + previously frozen conditioned medium (n=125, 8 repeats) following culture for 24-26 hours in medium containing FSH and serum. Groups 3 and 4 on the expansion scale (table 1) were counted as expanded. No significant difference was noted between groups (except mOOX) as determined by a Student's t-test ($p>0.05$). Results are means + s.e.m.

Figure 8.4. Photomicrographs of intact bovine cumulus oocyte complexes (COC) before (a) and after (b) incubation with FSH and serum for 24-26 hours, (c) bovine oocytectomised complexes (OOX) after incubation with serum and FSH for 24-26 hours. Nomarski optics (x120), bar represents 100μm

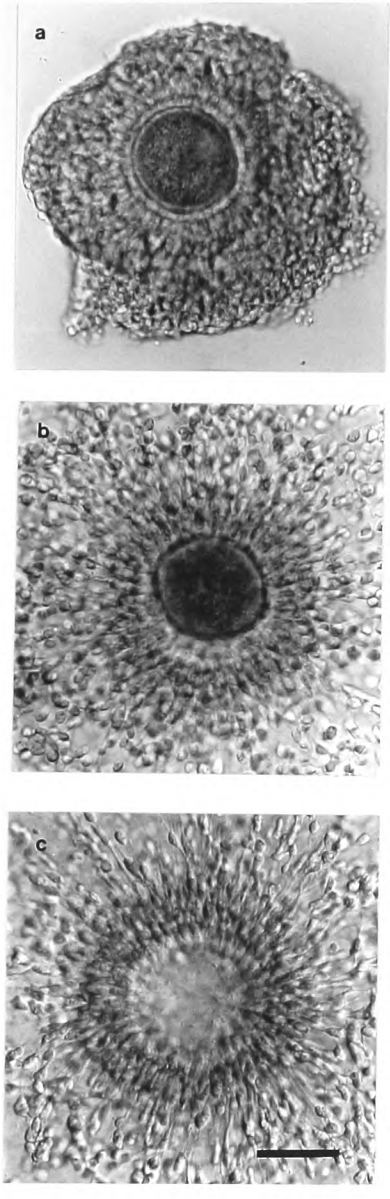
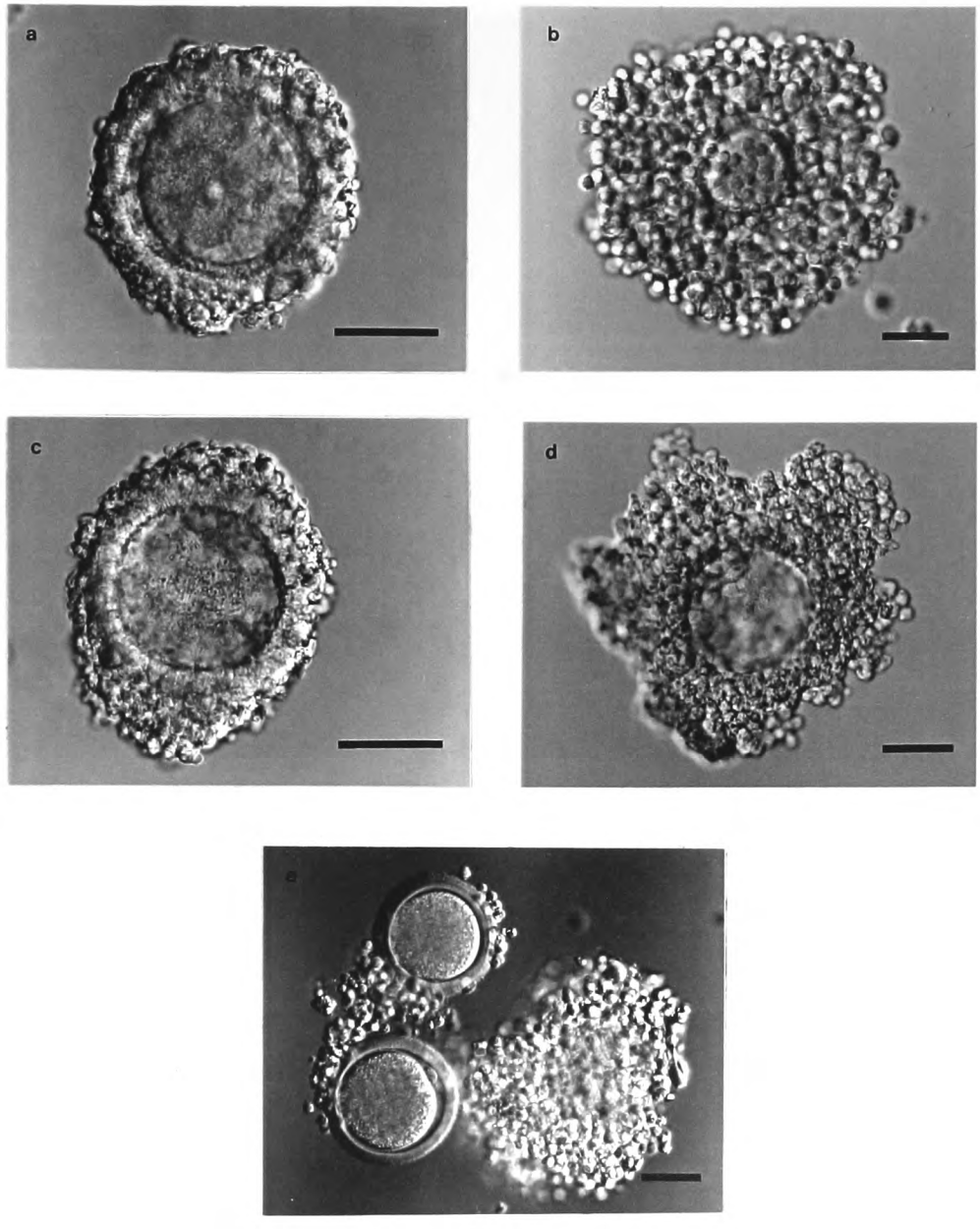

a
b
c

Figure 8.5. Photomicrographs of an intact murine cumulus oocyte complex (COC) before (a) and after (b) incubation with serum and FSH for 24-26 hours, murine oocytectomised complex (OOX) before (c) and after (d) incubation, murine OOX with denuded bovine oocytes following incubation (e). Bars represent 50μm. Nomarski optics, bar represents 50μm.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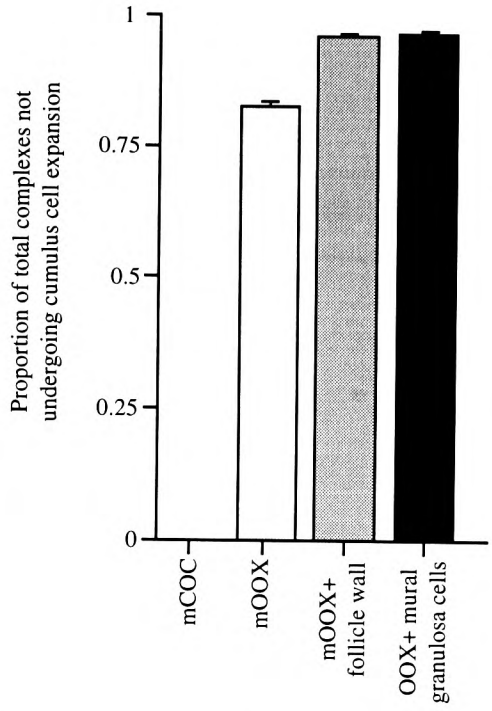

Figure 8.6. Histogram showing proportion of unexpanded murine cumulus oocyte complexes (mCOC), (n= 60, 6 repeats), murine oocytectomised complexes (mOOX) (n= 44, 6 repeats), mOOX + follicle wall (n= 114, 10 repeats) and mOOX + mural granulosa cells (n= 122, 11 repeats) following culture for 24-26 hr in medium containing FSH and serum. Groups 1 and 2 on the expansion scale (table 1) were counted as unexpanded. No significant differences were detected between groups (except mCOC) as determined by a Student's t-test ($p>0.05$). Results are mean ± s.e.m.

Treatment	Unexpanded complexes	Expanded complexes
(i) Bovine COC	8 (4.9)	156 (95.1)
(ii) Bovine OOX	14 (4.8)	275 (95.2)
(iii) Bovine OOX (2 hr delay)	0(0)	33(100)
(iv) Bovine COC (-FSH)	15 (71.4)	6 (28.6)

Table 8.2. The numbers of (i) bovine cumulus oocyte complexes (COC) (ii) bovine oocytectomised complexes (OOX) (iii) bovine OOX preincubated for 2 hours in dissection medium (iv) bovine COC matured in culture medium without FSH, which expand (groups 3 and 4 in expansion scale, table 1) or do not expand (groups 1 and 2 in expansion scale, table 1) following culture in maturation medium for 24-26. Results in brackets show the percentages. No significant difference was noted between groups (I) and (ii) ($p>0.05$) as determined using a χ^2 test

Treatment		Degree of Expansion			
	n	1	2	3	4
Intact cumulus oocyte complex	35	0	0	6(17)	29(83)
Clumps of cumulus cells	70	0	0	11(16)	59(84)
Clumps of cumulus cells + denuded bovine oocytes	60	0	0	10(17)	50(83)

Table 8.3. Table showing numbers of expanded cumulus oocyte complexes, clumps of cumulus cells and clumps of cumulus cells with denuded oocytes following 24-26 hours culture with FSH and serum. Results in brackets are percentages.

8.4. DISCUSSION

The experiments shown here demonstrate that bovine oocytes secrete a cumulus expansion enabling factor which allows murine cumulus cells (figures 8.3. and 8.5.) to expand in response to stimulation by FSH in the presence of serum. The zona pellucida, oocyte membrane or oocyte cytoplasm remaining after oocytectomy of bovine COC do not act as a store of the factor. Further, we have demonstrated that the factor is produced exclusively by the oocyte within the follicle. The factor can withstand freezing, allowing storage for further analysis. Bovine cumulus cells in common with porcine cumulus cells (Prochazka et al., 1991, Vanderhyden et al., 1993, Singh et al., 1993) do not require an oocyte or oocyte secreted factor during expansion as 95.2% of the bovine OOX complexes expanded without an oocyte (table 8.2 and figure 8.4). This result shows the factor secreted may have a different function in cattle than mice. Alternatively, it may point to differences in the process of cumulus cell expansion between species.

Figures 2 and 4 show that the presence of bovine oocytes provides the stimulus to enable murine OOX complexes to respond to FSH and undergo expansion (94%). This effect of the oocyte is not due to direct oocyte - cumulus cell contact as conditioning the medium with bovine oocytes caused 90% of the murine OOX complexes to expand (figure 8.3). Oocytectomy removes the contents of the oocyte, leaving behind the oocyte membrane, zona pellucida and cumulus cells (figure 8.2). The 3-dimensional organisation of the cumulus cells presents a more physiological situation for examining cumulus cell expansion than the use of isolated clumps of cumulus cells (Salustri et al., 1990a) where the gap junction communication from the peripheral cumulus cells to the zona pellucida is disrupted, although there may be confounding effects of the zona pellucida and remaining oocyte components.

Stimulation of the complexes with FSH was required for cumulus cell expansion. When intact bovine COC were cultured without FSH, some spontaneous cumulus cell expansion did occur (table 8.2) which may be partly due to low levels of FSH in the foetal calf serum. However, this was less than 30% of the total number of complexes and maximal expansion (group 4 in the expansion scale, table 8.1) was never observed.

In the results of experiment 1, it is possible that the zona pellucida, oocyte membrane or residual cytoplasm in the OOX complex may be acting as a store of the oocyte secreted factor thus allowing expansion of the bovine oocytectomised complexes. Isolated clumps of bovine cumulus cells did expand in the absence of the zona pellucida or any oocyte component (table 8.3), suggesting the independence of the bovine cumulus cells from the cumulus expansion enabling factor.

It was possible that other cells within the follicle may have produced a cumulus expansion enabling factor and the presence of these cells during culture of bovine OOX may account for cumulus cell expansion in the absence of an oocyte. Isolated mural granulosa cells did not stimulate cumulus cell expansion and are therefore unlikely to produce the cumulus expansion enabling factor (figure 8.6). Detachment of granulosa cells from the basement membrane has been shown to alter the production of a variety of factors (Furman et al., 1986, Maresh et al., 1990). However, when mural granulosa cells attached to the follicle wall were cultured with murine OOX, cumulus cell expansion was not stimulated. It therefore appears that the cumulus cell expansion enabling factor is produced exclusively by the oocyte within the follicle.

Pre-exposure of bovine cumulus cells to the cumulus expansion enabling factor prior to oocytectomy may allow cumulus cell expansion in the absence of the oocyte as shown in the rat (Vanderhyden, 1993). Preincubation of rat OOX complexes in Waymouth medium with foetal bovine serum and IBMX for at least 2 hours before stimulation by FSH caused a reduction in numbers of rat OOX

complexes undergoing cumulus cell expansion. However, the ability of the complexes to expand on addition of FSH and the cumulus expansion enabling factor was maintained (Vanderhyden, 1993). No such effect was seen in the pig OOX complexes which still underwent expansion in response to FSH 48 hours after oocytectomy. Bovine OOX complexes incubated for at least 2 hours before stimulation with FSH showed no reduction in their ability to undergo cumulus cell expansion (table 8.2).

From previous experiments it is known that the cumulus expansion enabling factor is unlikely to be a known growth factor as neither TGF-β1, PDGF, FGF, IGF-I or EGF replaced the oocyte secreted factor in stimulating murine cumulus cell expansion with FSH (Salustri et al., 1990b) even though certain growth factors can replace FSH and stimulate cumulus cell expansion (Salustri et al., 1990b, Lorenzo et al., 1994). Further, the factor appears to be specific to the oocyte (Buccione et al., 1990b). Taken together, these results show that stimulation of murine cumulus cell expansion requires a relatively specific factor(s). As bovine oocytes or bovine oocyte conditioned medium enables murine cumulus cells to expand, it is likely that both murine and bovine oocytes secrete a similar, if not identical factor(s).

Characterisation of the cumulus expansion enabling factor has been hampered by collection and storage difficulties (Eppig et al., 1993a). In vitro grown oocytes have been used as a potentially large source of cumulus expansion enabling factor (Eppig et al., 1993a). Although these oocytes were capable of stimulating cumulus cell expansion, the amount of cumulus expansion enabling factor produced by these oocytes was about half that of in vivo grown oocytes (Eppig et al., 1993a). In addition to the preliminary characterisation of the murine factor by Eppig et al., 1993a, we have now shown that from the abundant source of preovulatory oocytes from abattoir derived ovaries, the activity of the factor is still present after freezing and storage at -20°C for periods up to at least 1 month. This will allow quantities of

medium containing the factor to be stored for further purification and elucidation of its structure and function.

Here, a factor has been shown to be secreted by bovine oocytes which enables cumulus cell expansion by murine oocytes. Other paracrine factors may be involved in cumulus cell expansion. A more detailed understanding of these factors will be necessary for the improvement of current in vitro maturation and fertilisation systems.

CONCLUSIONS

In this study 2 areas in which current knowledge of the regulation of ovarian development is limited were examined: (i) factors affecting preantral and early antral follicles and (ii) the roles of the oocyte in follicular development. The study was conducted in cattle, a species of commercial importance and from which the findings are readily applicable to other large mammals.

Preantral follicles constitute the population from which antral follicles and eventually the preovulatory follicle are selected. The regulation of preantral follicle development is poorly understood. Culture of isolated preantral and early antral follicles provides the ideal opportunity to examine factors affecting their development.

The results of this thesis demonstrate for the first time that microdissection is a reliable method for the isolation of intact morphologically normal bovine preantral and early antral follicles. Through the avoidance of enzymes a suitable starting material for culture was provided.

Isolated follicles were used to devise a culture technique which supported bovine follicle and oocyte growth and would be suitable for examining their development. The key elements of the culture system were: volume of medium, serum and insulin, minimal number of medium changes and a substrate of collagen.

The isolation and culture system used enabled the most detailed study of the effect of FSH on the development of bovine preantral and early antral follicles to date. It is well established that as follicles enter the antral stage of development they become increasingly responsive to FSH. This study confirmed these observations by the addition of FSH to the cultures of isolated preantral and early antral follicles. FSH was found to affect the growth of large preantral and early antral follicles but not preantral follicles, therefore the effect of FSH depended on the stage of follicle

examined. The agreement of our observations with in vivo studies confirms the suitability of this system for examining bovine follicular development.

The controlled conditions of culture gave a new and detailed insight into the role of FSH dosage on the development of large preantral/early antral bovine ovarian follicles. FSH stimulates follicle growth, but the dosage was of critical importance as high concentrations reduced granulosa cell proliferation, oocyte growth and oocyte quality. This has important implications for techniques such as superovulation, which by administration of high doses of gonadotrophins, whilst promoting final oocyte development may adversely affect the development of smaller follicles and have long term effects on fertility.

The effect of FSH on follicle size was due to an increase in intercellular spacing and, as antral cavities were neither maintained or formed during culture, this may be analogous to antrum development. The mechanisms underlying antral cavity formation are unclear and requires further investigation for which the approach used here will be useful.

The oocyte regulates a number of aspects of murine follicular development. Its roles in bovine follicular development have yet to be established. In this study, oocyte localised granulosa cell proliferation was observed in cultured bovine preantral and early antral follicles but only if a healthy oocyte was present. This indicates a possible role of the oocyte in directing granulosa cell proliferation and differentiation. The intensity of oocyte localised proliferation was reduced by high FSH doses, further confirming its dose dependent inhibitory effect on follicular development. The nature of the oocyte effect on proliferation is not known and further studies are required to investigate this.

To further examine the role of the oocyte in directing somatic cell function, its effect on cumulus cell expansion was examined. Using intact bovine cumulus oocyte complexes or oocytectomised complexes (OOX), it was

demonstrated that the oocyte is not essential for bovine cumulus cell expansion. Murine OOX complexes, which require an oocyte secreted factor for cumulus expansion, expanded in the presence of bovine oocytes Thus some factor secreted exclusively by the oocyte enabled expansion of murine OOX complexes. The purpose of the factor in bovine follicle development is unknown. Furthermore, this highlights a species specific difference in the regulation of somatic cell function by the oocyte, underlining the need for (i) more detailed studies in larger mammals and (ii) caution when interpreting studies of murine follicular development.

Apoptosis is thought to be how large follicles become atretic. It is not known if apoptosis underlies atresia in preantral follicles. Isolation of distinct follicle classes allowed apoptosis in preantral to early antral follicles to be examined in detail. The work presented here demonstrates that follicles isolated and selected for culture did not show apoptotic DNA fragmentation, confirming their suitability as a starting material for in vitro studies. Culture of follicles in conditions which induce apoptosis in isolated granulosa cells, did not result in apoptosis. It is possible that the oocyte has a modulating influence on follicular apoptosis or that cell death may occur by an alternative mechanism.

A deeper understanding of the regulation of follicular development will enable further advances in assisted reproductive technology. In particular, further improvements to the immature follicle isolation and culture method devised here may enable the production of large numbers of developmentally competent oocytes. The enormous advantages of such a system are obvious: increased offspring from animals of high genetic merit, supply of homogenous oocytes for embryo production or new technologies (e.g. nuclear transfer), preservation of rare breeds and increased rates of genetic improvement through reduced generation interval by growing oocytes isolated from foetal ovaries. Additionally, studies of follicular development of cattle follicles are more readily applicable to other large mammals (than previous

murine systems) opening new opportunities for examining clinical infertility or ovarian toxicology.

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A. APPENDIX

A.1. MACRO PROGRAMMES USED FOR IMAGE ANALYSIS MEASUREMENTS

During image analysis of the histological sections, macro programmes can be assigned to a number of keys to automatically perform a series of tasks. The following macros were used to (i) capture an image of the follicle section to be stored in the computer and (ii) to measure the area of the granulosa cells within the follicle selected by the area selection tool. The macros were written using the inbuilt text editor of NIH image. Full details of the programming language can be found in the instruction manuals which accompany the programme when downloaded from the internet (see chapter 5.2.).

A.1.1. Programme for capturing image of section from microscope

```
macro 'CaptureImage [F1]';
begin;
StartCapturing;
StopCapturing;
EnhanceContrast;
SetScale(1.925,'μm');
SaveAs('');
end
```

A.1.2. Programme for measuring total granulosa cell area

```
macro 'Cell Area [F5]';
begin;
```

```
ResetCounter;
SetDensitySlice(65,255);
Beep;
PutMessage('Adjust density slice');
end
```

A.2. JOURNAL OF REPRODUCTION AND FERTILITY 1994; ABSTRACT SERIES 13:23

BOVINE CUMULUS CELL EXPANSION IS NOT DEPENDANT ON AN OOCYTE PRODUCED FACTOR EVEN ALTHOUGH ONE IS SECRETED BY THE OOCYTE. J.H.Ralph[1], E.E.Telfer[2] & I.Wilmut Roslin Institute, Roslin, EH25 9PS ,[2]Institute of Ecology and Resource Management, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JG

The roles of the oocyte and communication between follicular somatic cells in follicular development and function are increasingly being recognised as important factors. Here we examined how the oocyte may be involved in bovine cumulus cell expansion. 1. Bovine cumulus oocyte complexes (COC) were obtained by puncturing antral follicles and oocytectomised complexes (OOX) were produced by micro surgical oocyte removal. OOX or COC were matured in the presence of foetal calf serum and hFSH (6mIU/ml) for 24 hours and the degree of expansion examined. The oocyte is not essential for bovine cumulus expansion to occur as expansion occurred both in the OOX and COC. 2. Murine OOX complexes from eCG primed 35-40 day old C57BL6/CBA F_1 hybrids (known to require the presence of an oocyte secreted factor for cumulus expansion) were cultured with or without denuded bovine oocytes (1 oocyte/<micro>l). Murine OOX complexes only expanded in the presence of denuded bovine oocytes. Thus some factor secreted by bovine oocytes permitted expansion of murine OOX complexes. 3. To determine whether the factor is secreted, murine OOX were cultured with or without media conditioned by bovine oocytes (1 oocyte/<micro>l for 4 hours). Significant expansion of murine OOX occurred only in the presence of media conditioned by bovine oocytes. This shows that the cumulus expansion enabling effect of bovine oocytes is released into the surrounding media. 4. The factor can withstand freezing.

Thus it is concluded that although bovine COC do not require the presence of an oocyte for cumulus cell expansion, bovine oocytes do secrete a factor which enables cumulus expansion to occur in murine OOX complexes. A better understanding of the role of the oocyte and communication between somatic cells may help improve current in vitro maturation and fertilisation systems.

[1]Supported by the Meat and Livestock Commission.

A.3. JOURNAL OF REPRODUCTION AND FERTILITY 1995; ABSTRACT SERIES 15:6

IN VITRO GROWTH OF BOVINE PREANTRAL FOLLICLES AND THE INFLUENCE OF FSH ON FOLLICULAR AND OOCYTE DIAMETERS. J.H.Ralph, I.Wilmut and E.E.Telfer[1]
Roslin Institute, Roslin, Midlothian, EH25 9PS, [1]Institute of Ecology and Resource Management, University of Edinburgh, West Mains Road, Edinburgh, EH9 3JG

Culture of preantral follicles isolated from livestock has potentially important applications in agriculture and research. The aim of this study was to develop a technique for the isolation and culture of bovine preantral follicles and to investigate the influence of FSH on their growth in vitro. Cortical slices were taken from abattoir derived bovine ovaries. Micro-dissection with fine needles was used to isolate large preantral follicles (approximately 170-180 microns). Groups of 4 follicles were cultured on collagen for 5 days at 39°C in a humidified atmosphere of 5% CO_2 in 1ml TCM199 with pyruvate, insulin and transferrin. Follicle and oocyte diameters were measured on days 0,1,3 and 5 of culture. Tritiated thymidine (0.185MBq/ml) was added on the penultimate day of culture. On day 5, follicles were fixed, embedded in wax and sectioned. Following autoradiography, the sections were stained with haemotoxylin and eosin. Follicular morphology was analysed by microscopy and tritiated thymidine incorporation examined using image analysis. In the second experiment 0,10,100 or 1000mU/ml of ovine FSH was included in the culture media. The results show that 1. follicular and oocyte diameters increased throughout the duration of culture 2. follicular morphology was maintained 3. tritiated thymidine incorporation by the granulosa cells was observed and 4. when FSH was included in the culture media follicle diameter was greater although no significant differences were observed in oocyte diameter. In conclusion, large bovine preantral follicles can be isolated, cultured and grown in vitro . Further, the follicles have been shown to respond to FSH by an increase in follicular size, demonstrating that this culture system is suitable for investigating follicular development.

J.H.Ralph acknowledges the support of the Meat and Livestock Commission and MAFF

A.4. PROCEEDINGS OF THE 1ST UK NATIONAL OVARIAN WORKSHOP 1995, ROYAL FREE HOSPITAL SCHOOL OF MEDICINE, LONDON. ABSTRACT 1.

BOVINE OVARIAN FOLLICLE GROWTH IN VITRO: THE EFFECT OF FSH ON PREANTRAL FOLLICLES OF DIFFERENT SIZES. John H. Ralph, Ian Wilmut and Evelyn E.Telfer*. Division of Development and Reproduction, Roslin Institute, Roslin, Midlothian, EH25 9PS. *School of Agriculture, Institute of Ecology and Resource Management, University of Edinburgh, Kings Buildings, West Mains Road, Edinburgh

Isolation and culture of preantral follicles may provide an abundant source of competent oocytes for assisted reproduction. Further, in vitro follicular development would allow an insight into the regulation of folliculogenesis. Follicle stimulating hormone (FSH) is one of the main factors involved in the control of follicle development. In this study, the influence of FSH on the development of different sizes of bovine preantral follicles will be examined.

Fine cortical slices were taken from the surface of abattoir derived bovine ovaries and placed in dissection medium. Using a dissection microscope fitted with a heated stage, 25 guage needles were used to dissect small (~120mm), medium (~180mm) and large (~220mm) preantral follicles. Selected follicles were cultured in groups of 4, on collagen, in 1ml of TCM199 supplemented with insulin, transferrin, pyruvate and 10% heat inactivated foetal calf serum in a humidified atmosphere of 5% CO_2 at 39°C. The effect of the addition of 0 or 25mIU/ml of ovine FSH was examined. Follicle and oocyte diameters were measured on days 0, 1, 3 and 5 of culture using an inverted microscope. Tritiated thymidine was added on the penultimate day of culture. On day 5, follicles were fixed, embedded in paraffin wax and sectioned. The sections were dipped in photographic emulsion and exposed for 3.5 days, developed and lightly stained with haemotoxylin and eosin. Follicular morphology was examined by light microscopy. Granulosa cell proliferation was determined by viewing the sections under dark field and counting silver grain clusters.

J.H.Ralph acknowledges the support of the Meat and Livestock Commission and MAFF

A.5. BIOLOGY OF REPRODUCTION 1996, 54:SUPPLEMENT 1, ABSTRACT 5

THE EFFECT OF FSH ON BOVINE PREANTRAL TO EARLY ANTRAL OVARIAN FOLLICLE GROWTH IN VITRO. JH Ralph,*[1] I Wilmut,*[1] and EE Telfer[2], Division of Development and Reproduction, Roslin Institute, Roslin, Midlothian, U.K.[1], Institute of Ecology and Resource Management, School of Agriculture Building, University of Edinburgh, Edinburgh, U.K.[2]

Isolation and culture of immature ovarian follicles may provide an abundant source of oocytes for assisted reproduction. Further, in vitro follicular development would allow a deeper insight into the regulation of folliculogenesis. Follicle stimulating hormone (FSH) is one of the main factors involved in the control of follicular development. Here the effect of FSH on bovine preantral to early antral ovarian follicle growth was examined.

Preantral (105-160μm), large preantral/early antral (150-200μm) and antral (190-250μm) follicles were isolated by microdissection of cortical slices from abattoir derived bovine ovaries. Groups of 4 follicles were cultured for 5 days on a collagen matrix in 1ml of medium (TCM199, pyruvate, insulin, transferrin and 10% heat inactivated foetal calf serum) with or without 25mIU of ovine FSH at 39°C in a humidified atmosphere of 5% CO_2. Follicle and oocyte diameters were measured on days 0, 1, 3 and 5 of culture. Tritiated thymidine was added 24 hours before the end of culture for assessment of granulosa cell proliferation. On day 5, follicles were fixed and sectioned for histology and autoradiography.

In all treatments, follicles and oocytes increased in size during culture. By day 5 FSH significantly stimulated the growth of the large preantral/early antral follicles ($\bar{x}$=222.9μm ± 3.8μm without FSH, $\bar{x}$=238.4μm ± 4.9μm with FSH, $p<0.05$) and antral follicles ($\bar{x}$=275.2μm ± 5.5μm without FSH, $\bar{x}$=313.1μm ± 8.4μm with FSH, $p<0.05$), but not that of the preantral class. FSH did not effect oocyte diameter during culture. Follicles appeared morphologically normal after culture, with oocyte in the germinal vesicle stage, intact basement membrane and few pyknotic cells. FSH stimulated granulosa cell proliferation in preantral follicles. In many follicles proliferation was localised to the granulosa cell layers nearest the oocyte.

It is concluded that this isolation method and culture system support short term growth of bovine preantral to early antral follicles. We have shown that preantral follicles respond to FSH by an increase in granulosa cell proliferation. FSH stimulated the growth of large preantral/early antral and antral follicles, but the current protocol was unable to detect differences in thymidine uptake. Oocytes increased in

size in all treatment groups but no effect of FSH was observed. (Supported by a UK Meat and Livestock Commission Studentship and the Ministry of Agriculture, Fisheries and Food)

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Bovine Cumulus Cell Expansion Does Not Depend on the Presence of an Oocyte Secreted Factor

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ABSTRACT Communication between the oocyte and its somatic cells has been shown to be important in oocyte development. Here we examined how the oocyte may be involved in bovine cumulus cell expansion. Intact bovine cumulus oocyte complexes (COC) were obtained by puncturing antral follicles. From the intact COC, oocytectomised complexes (OOX) were produced by micro surgical removal of the oocyte. Clumps of cumulus cells (CC) were obtained by micro-dissection. Intact or OOX complexes or CC were matured in the presence of fetal calf serum and hFSH (6 mIU/ml) for 24 hr and the degree of expansion measured. The presence of the oocyte is not essential to allow bovine cumulus expansion to occur as expansion occurred in all groups. Murine OOX complexes from eCG primed 35–40-day-old C57BL6/CBA F_1 hybrids (known to require the presence of an oocyte secreted factor for cumulus expansion) were cultured with or without denuded bovine oocytes (1 oocyte/μl). Murine OOX complexes expanded only in the presence of denuded bovine oocytes. Thus some factor produced by bovine oocytes enabled expansion of murine OOX complexes. To determine whether the factor is secreted by bovine oocytes, murine OOX were cultured with or without media conditioned by bovine oocytes (1 oocyte/μl for 4 hr). Significant expansion of murine OOX occurred in media conditioned by bovine oocytes. This shows that the cumulus expansion enabling effect of bovine oocytes is released into the surrounding media. Media conditioned by bovine oocytes and then frozen for up to 1 month showed that the activity by the factor can withstand freezing.

Key Words: Paracrine factors, Oocyte development, Cumulus expansion enabling factor, In vitro maturation, In vitro fertilization

INTRODUCTION

The oocyte and companion somatic cells comprising the follicular unit maintain a close association throughout development from primordial to preovulatory stages. The intercommunication between the oocyte and the somatic cells is recognised as an important component in growth and differentiation (Salustri et al., 1990a,b; Buccione et al., 1990b; Eppig et al., 1993a,b; Vanderhyden et al., 1992; for reviews see Buccione et al., 1990a; Eppig, 1991). In the mouse this communication has been shown to be important in cumulus cell expansion.

Cumulus cell expansion occurs around the time of ovulation (precise timing is species dependant, Motlik et al., 1986), where in response to the gonadotrophin surge, the cumulus cells surrounding the oocyte secrete hyaluronic acid forming a gelatinous matrix around the oocyte (Dekel and Kraicer, 1978). The gelatinous matrix is thought to be important in pick up of the complex by the oviductal fimbrae and also in fertilisation (Meizel, 1985; Salustri et al., 1989). Moreover, expansion disrupts gap-junctional communication between the cumulus cells and the oocyte (Motlik et al., 1986) and has been implicated in the control of meiotic arrest (Downs, 1993; Eppig and Downs, 1984). Cumulus cell expansion of isolated intact cumulus oocyte complexes (COC) can be stimulated in vitro and the mature complex used for in vitro fertilization (Madison et al., 1992). It is easily recognised in vitro, where the arrangement of cumulus cells changes from a tightly compact configuration around the oocyte to a larger glistening mass, within which individual isolated cumulus cells can be seen (Eppig, 1980a).

The oocyte has been shown to play an important role in cumulus cell expansion in the mouse and rat (Buccione et al., 1990b; Salustri et al., 1990a,b; Vanderhyden, 1993). In response to FSH and in the presence of serum, murine cumulus cells undergo hyaluronic acid synthesis and expansion only if fully grown, meiotically component oocytes or media conditioned by these oocytes are present (Buccione et al., 1990b; Salustri et al., 1990a,b). Therefore an oocyte or oocyte secreted factor(s) is essential for murine cumulus cell expansion (Buccione et al., 1990b; Salustri et al., 1990a). Conditioning media with other cell types (granulosa cells, sertoli cells, spermatozoa or fibroblasts) has failed to enable cumulus cell expansion (Buccione et al., 1990b). The factor, termed the "cumulus expansion enabling factor", is secreted specifically by the oocyte (Buccione et al., 1990b). In the pig, although such a factor is secreted by the oocyte, it does not appear to be essential

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for expansion (Prochazka et al., 1991; Singh et al., 1993; Vanderhyden, 1993). It is not known whether bovine oocytes secrete a cumulus expansion enabling factor and if such a factor is required for expansion.

Simple characterization of the murine cumulus expansion enabling factor was carried out by Eppig et al. (1993a). So far, it is known that it has a nominal molecular weight of 100–300 kd and is likely to be a protein or dependent on a protein for its activity. Further analysis of the factor has been complicated by: (1) limited collection of large quantities of the factor due to the small number of fully grown oocytes obtained (Eppig et al., 1993a), and (2) instability of the factor, which loses its activity after several hours (Eppig et al., 1993a). A method of collection and storage of the factor maintaining activity would help in its elucidation. The aims of this study were, therefore: (1) to determine whether bovine cumulus cells require the presence of an oocyte or oocyte secreted factor for cumulus cell expansion to occur, (2) to establish if bovine oocytes produce a factor that enables cumulus cell expansion in the mouse, and (3) to devise a simple method for the storage of bovine oocyte cumulus expansion enabling factor.

MATERIALS AND METHODS

Experiment 1: Requirement of Bovine Oocytes for Cumulus Cell Expansion

Isolation of cumulus oocyte complexes (COC). Bovine ovaries were obtained from an abbattoir at 30°C, washed with industrial methylated spirits, and placed in PBS (Unipath, Basingstoke, U.K.) with 50 mg/l Gentamycin (Sigma Chemical Co., Poole, U.K.). 2–6 mm follicles were aspirated using a 25G needle and syringe. The follicular fluid collected was allowed to settle for 15 min and the overlying fluid drawn off. The residue was resuspended in dissection media (M199 (cat. no. 21180-013 GIBCO-BRL, Life Technologies, Paisley, U.K.), 0.03 M Hepes (Sigma), 0.13 mM Kanomysin Monosulphate (Sigma), 10% fetal calf serum (Globepharm, Surrey, U.K.), and 50 μM IBMX (Sigma)), and good quality COC complexes (fully cumulus enclosed with evenly pigmented oocyte cytoplasm) were selected under the dissecting microscope.

Microsurgical oocyte content removal (oocytectomy). Microsurgical removal of the contents of the oocyte was performed as described previously. (Buccione et al., 1990b; Prochazka et al., 1991). Briefly, using micromanipulators with a holding pipette (20 μm diameter hole) and a fine glass needle, the complex is held under negative pressure and the oocyte pierced through both sides of the zona pellucida. The contents of the oocyte are then aspirated through the holding pipette on removal of the glass needle, resulting in temporary deformation of the zona pellucida. Almost all of the oocyte contents are removed in this way, leaving an essentially intact complex without the oocyte—the oocytectomised complex (OOX) (see Fig. 1). Occasionally this procedure resulted in loss of the cumulus cells and only OOX complexes with at least three complete cumulus cell layers were used in this study.

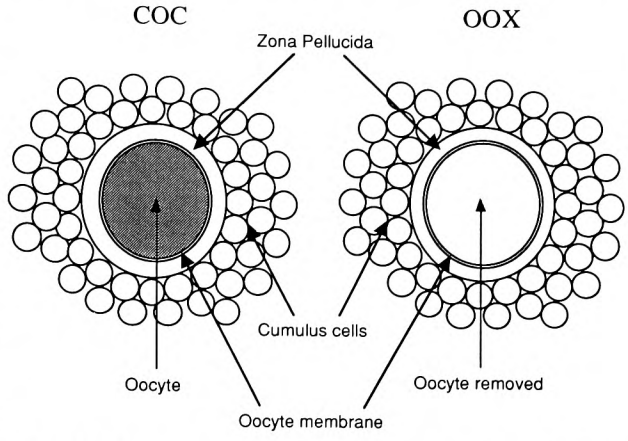

Fig. 1. Schematic diagram showing cumulus oocyte complex (COC) and oocytectomized complex (OOX). Oocytectomy removes the oocyte contents leaving the oocyte membrane, zona pellucida and arrangement of cumulus cells intact.

Measurement of cumulus cell expansion. Intact and OOX complexes were rinsed twice in the culture media (M199 GIBCO-BRL, Life Technologies), 0.02 M Hepes (Sigma), 0.026 M sodium bicarbonate (Sigma), 10% fetal calf serum (Globepharm), and 6 mIU FSH (Pergonal, Serono Laboratories, Welwyn Garden City, U.K.) to remove any IBMX. Groups of COC or OOX complexes were added to 50 μl drops of culture media under paraffin oil and incubated for 24 hr to allow maximal expansion at 39°C and 5% CO_2. At 24–26 hr, the degree of expansion was scored using a qualitative scale (Table 1) and the results analysed using a chi-square test.

Experiment 2: Role of Zona Pellucida, Oocyte Membrane, or Residual Oocyte Cytoplasm in Bovine Cumulus Cell Expansion

It is possible that the zona pellucida, oocyte membrane, or residual oocyte cytoplasm may provide the bovine OOX complex with the correct stimulus for cumulus cell expansion to occur. To examine this possibility, groups of 10 clumps of cumulus cells (without any oocyte component or zona pellucida) were microdissected from COC and incubated at 39°C, 5% CO_2 in 50 μl drops of culture medium under paraffin oil for 24–26 hr. Expansion was then scored as before.

Experiment 3: Production of Factor That Enables Murine Cumulus Cell Expansion

Murine OOX complexes are known to require the presence of an oocyte secreted factor before cumulus cell expansion can occur (Buccione et al., 1990b; Salustri et al., 1990a). In this experiment the production of such a factor by bovine oocytes was analysed.

Isolation and preparation of intact and OOX murine complexes and denuded bovine oocytes. Ovaries were removed from 35-day-old, 48-hr eCG primed C57BL6/CBA F1 mice and placed in M2 media (Hogan

TABLE 1. Scale Used to Measure Degree of Cumulus Cell Expansion

1	2	3	4
No expansion	Minimal expansion; slight glistening of complex	Expansion; all layers of cumulus cells expand except the corona radiata	Maximum expansion; all layers of cumulus cells expand

et al., 1986) with 50 μM IBMX. Large follicles were punctured under the dissecting microscope using 25G needles and murine COC released. Oocytectomy was performed as before using murine COC in 200 μl drops of M2 media and IBMX under paraffin oil.

Bovine COC were isolated as before. Denuded bovine oocytes were prepared by repeated pipetting using a 1 ml pipette to remove almost all the cumulus cells (Eppig and Downs, 1984; Vanderhyden et al., 1993).

Assessment of cumulus cell expansion. After rinsing twice in culture media to remove the IBMX, groups of 9–15 mouse COC, mouse OOX, or mouse OOX plus 50 denuded bovine oocytes were added to 50 μl drops of culture media under paraffin oil. The microdrops were cultured for 24–26 hr and examined for cumulus cell expansion. Results were analysed using Students *t*-test.

Experiment 4: Examination of Secretion of Bovine Cumulus Expansion Enabling Factor

The aim of this experiment was to determine if the beneficial effect of the bovine oocytes is mediated by direct contact of the bovine oocytes with the murine OOX or by a secreted factor. Denuded bovine oocytes were prepared as before. Culture media was conditioned by adding denuded bovine oocytes at a concentration of 1 oocyte/μl and incubating for 4 hr at 39°C, 5% CO_2. After this time, the conditioned media was collected and used to prepare 50 μl drops under paraffin oil. Between 9 and 15 murine OOX were added to each drop and expansion examined after 24–26 hr. The results were analysed using Student *t*-test.

Experiment 5: Storage of Conditioned Media

To determine whether the media conditioned by bovine oocytes could be stored frozen while maintaining its cumulus expansion enabling activity, aliquots of conditioned media (prepared as above) were frozen (−20°C) immediately after collection for up to 1 month. As required, aliquots were heated to 39°C and divided into 50 μl drops. Between 9 and 15 murine OOX were added to each drop and examined for expansion at 24–26 hr, and the results analysed using Student *t*-test.

RESULTS

Experiment 1: Requirement of Bovine Oocyte for Cumulus Cell Expansion

Cumulus cell expansion (groups 3 and 4 in the expansion scale, Table 1) was observed in both the intact COC and OOX complexes (Table 2, $P > 0.05$, Fig. 3). The presence of bovine oocytes during cumulus cell expansion does not therefore appear to be necessary in cattle.

TABLE 2. Numbers of Bovine Cumulus Oocyte Complexes (COC), Bovine Oocytectomized Complexes (OOX) That Expand (groups 3 and 4 in expansion scale, Table 1) or Do Not Expand (groups 1 and 2 in expansion scale, Table 1) Following Culture for 24–26 Hr in the Presence of Serum and FSH*

	Unexpanded complexes	Expanded complexes
Bovine COC	8(4.9)	156(95.1)
Bovine OOX	14(4.8)	275(95.2)

*Results in brackets show the percentages. No significant difference was noted between groups ($P > 0.05$) as determined using a χ^2 test.

TABLE 3. Numbers of Expanded Cumulus Oocyte Complexes, Clumps of Cumulus Cells, and Clumps of Cumulus Cells With Denuded Oocytes Following 24–26 Hr Culture With FSH and Serum*

		Degree of expansion			
Treatment	n	1	2	3	4
Intact cumulus oocyte complex	35	0	0	6(17)	29(83)
Clumps of cumulus cells	70	0	0	11(16)	59(84)
Clumps of cumulus cells + denuded bovine oocytes	60	0	0	10(17)	50(83)

*Results in brackets are percentages.

Experiment 2: Role of Zona Pellucida, Oocyte Membrane, or Oocyte Cytoplasm in Cumulus Cell Expansion

Culturing clumps of bovine cumulus cells for 24–26 hr with or without bovine oocytes had no effect on their ability to undergo cumulus cell expansion (Table 3). This suggests that during bovine cumulus cell expansion, the presence of the oocyte, structural components of the oocyte, or zona pellucida is not required.

Experiment 3: Production of Factor That Enables Murine Cumulus Cell Expansion

Removal of murine oocyte contents prevents cumulus cell expansion of the complexes following culture (Figs. 2 ($P < 0.05$), 4c,d) as has been shown previously (Buccione et al., 1990b). The presence of bovine oocytes in culture permits cumulus cell expansion of murine OOX complexes as shown in Figures 2 and 4e.

Experiment 4: Examination of Secretion of Bovine-Produced Cumulus Expansion Enabling Factor

Bovine oocyte conditioned media allowed cumulus cell expansion in murine OOX complexes (Fig. 2, $P > 0.05$). As no direct contact between bovine oocytes

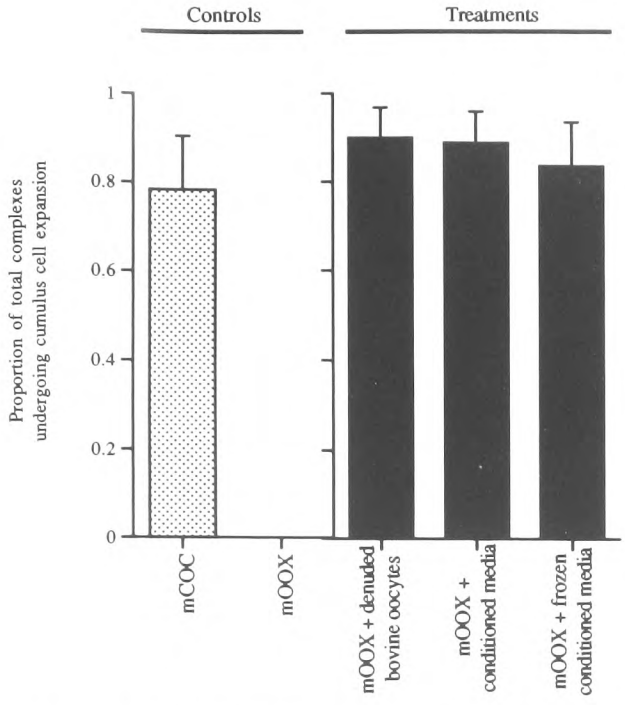

Fig. 2. Graph showing proportion of expanded murine cumulus oocyte complexes (mCOC), (n = 126, 8 repeats), murine oocytectomized complexes (mOOX) (n = 49, 5 repeats), mOOX + denuded bovine oocytes (n = 56, 6 repeats), mOOX + bovine oocyte conditioned media (n = 59, 6 repeats) and mOOX + previously frozen conditioned media (n = 125, 8 repeats) following culture for 24–26 hr in media containing FSH and serum. Groups 3 and 4 on the expansion scale (Table 1) were counted as expanded. No significant difference was noted between groups (except mOOX) as determined by Student's *t*-test ($P > 0.05$). Results are means + s.e.m.

and the murine OOX complexes was required to stimulate expansion, this suggests that bovine oocytes condition the culture media by secreting a factor that enables expansion to occur.

Experiment 5: Storage of Conditioned Media

Frozen/thawed bovine oocyte conditioned media still permits murine OOX complexes to undergo cumulus cell expansion (see Fig. 2, $P > 0.05$). The cumulus expansion enabling factor secreted by bovine oocytes can therefore be stored frozen, maintaining its expansion promoting ability for a period of up to at least 1 month. This may allow stocks of the media to be collected and stored for further analysis.

DISCUSSION

The experiments shown here demonstrate that bovine oocytes secrete a cumulus expansion enabling factor that allows murine cumulus cells (Figs. 2, 4) to expand in response to stimulation by FSH in the presence of serum. The zona pellucida, oocyte membrane, or oocyte cytoplasm remaining after oocytectomy of bovine COC does not act as a store of the factor. Further, we have demonstrated that the factor can withstand

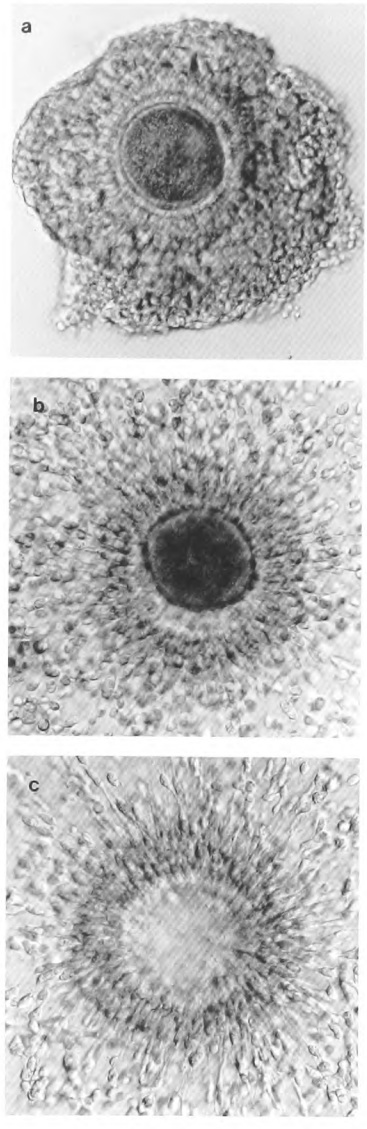

Fig. 3. Photomicrographs of intact bovine cumulus oocyte complexes (COC) before (**a**) and after (**b**) incubation with FSH and serum for 24–26 hr, (**c**) bovine oocytectomized complexes (OOX) after incubation with serum and FSH for 24–26 hr. Nomarski optics (×120).

freezing, allowing storage for further analysis. Bovine cumulus cells in common with porcine cumulus cells (Prochazka et al., 1991; Singh et al., 1993; Vanderhyden et al., 1993) do not require an oocyte or oocyte secreted factor during expansion as 95.2% of the bovine OOX complexes expanded without an oocyte (Table 2, Fig. 3). This result shows the factor secreted may have a different function in cattle than mice. Alternatively, it may point to differences in the process of cumulus cell expansion between species.

Figures 2 and 4 show that the presence of bovine oocytes provides the stimulus to enable murine OOX complexes to respond to FSH and undergo expansion (94%). This effect of the oocyte is not due to direct

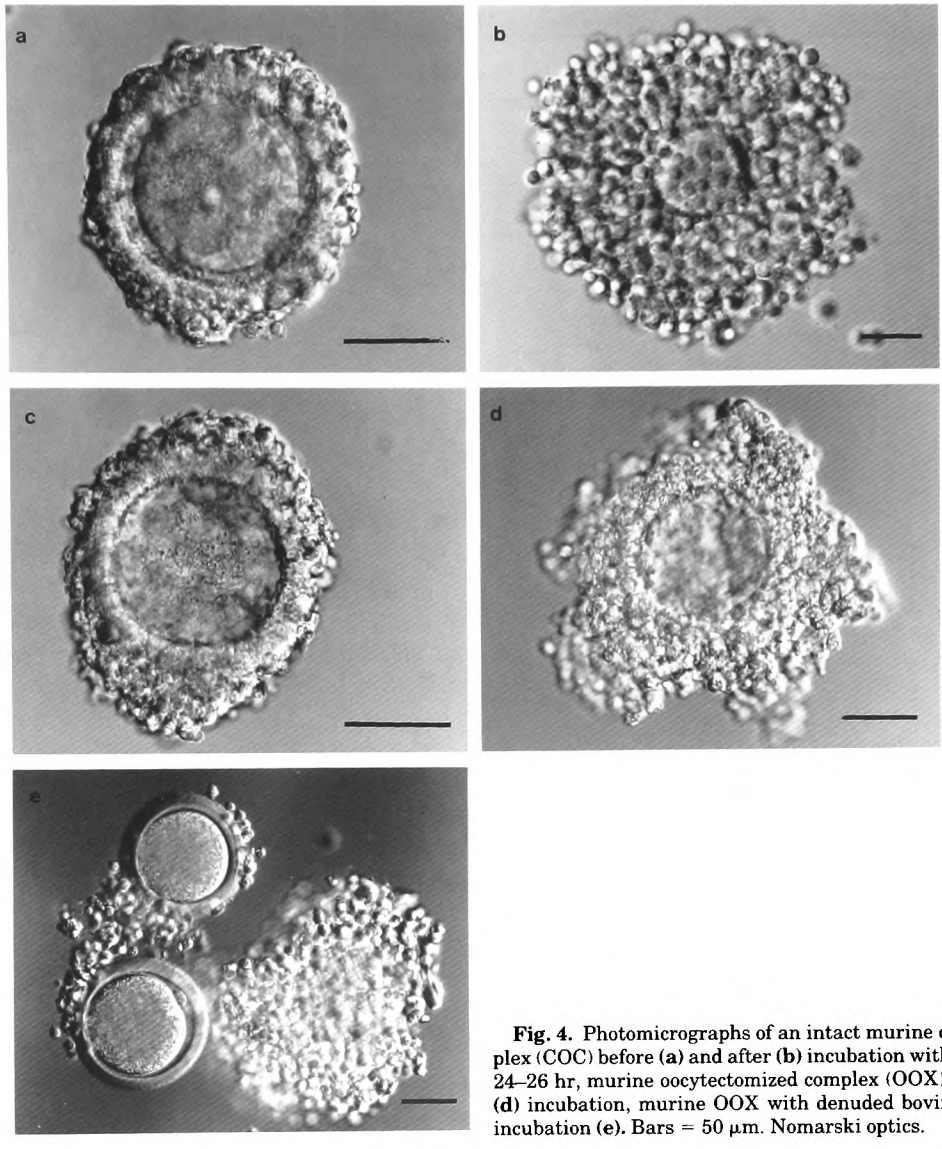

Fig. 4. Photomicrographs of an intact murine cumulus oocyte complex (COC) before (**a**) and after (**b**) incubation with serum and FSH for 24–26 hr, murine oocytectomized complex (OOX) before (**c**) and after (**d**) incubation, murine OOX with denuded bovine oocytes following incubation (**e**). Bars = 50 μm. Nomarski optics.

oocyte-cumulus cell contact as conditioning the media with bovine oocytes caused 90% of the murine OOX complexes to expand (Fig. 2). Oocytectomy removes the contents of the oocyte, leaving behind the oocyte membrane, zona pellucida, and cumulus cells (Fig. 1). The three-dimensional organisation of the cumulus cells presents a more physiological situation for examining cumulus cell expansion than the use of isolated clumps of cumulus cells (Salustri et al., 1990a) where the gap junction communication from the peripheral cumulus cells to the zona pellucida is disrupted, although there may be confounding effects of the zona pellucida and remaining oocyte components.

Stimulation of the complexes with FSH was required for cumulus cell expansion. In preliminary experiments where intact bovine COC and OOX complexes were cultured without FSH, some spontaneous cumulus cell expansion did occur. However, this was always <30% of the total number of complexes, and maximal expansion (group 4 in the expansion scale, Table 1) was never observed.

In the results of Experiment 1, it is possible that the zona pellucida, oocyte membrane, or residual cytoplasm in the OOX complex may be acting as a store of the oocyte secreted factor, thus allowing expansion of the bovine oocytectomised complexes. Isolated clumps of bovine cumulus cells did expand in the absence of the zona pellucida or any oocyte component (Table 3), suggesting the independence of the bovine cumulus cells from the cumulus expansion enabling factor.

Pre-exposure of bovine cumulus cells to the cumulus expansion enabling factor prior to oocytectomy may allow cumulus cell expansion in the absence of the oocyte as shown in the rat (Vanderhyden, 1993). Preincubation of rat OOX complexes in Waymouth media with fetal bovine serum and IBMX for at least 2 hr before stimulation by FSH caused a reduction in numbers of rat OOX complexes undergoing cumulus cell expan-

sion. However, the ability of the complexes to expand on addition of FSH and the cumulus expansion enabling factor was maintained (Vanderhyden, 1993). No such effect was seen in the pig OOX complexes, which still underwent expansion in response to FSH 48 hr after oocytectomy. Bovine OOX complexes incubated for at least 2 hr before stimulation with FSH showed no reduction in their ability to undergo cumulus cell expansion (unpublished observations).

From previous experiments, it is known that the cumulus expansion enabling factor is unlikely to be a known growth factor as neither TGF-β1, PDGF, FGF, IGF-1, or EGF replaced the oocyte secreted factor in stimulating murine cumulus cell expansion with FSH (Salustri et al., 1990b), even though certain growth factors can replace FSH and stimulate cumulus cell expansion (Lorenzo et al., 1994; Salustri et al., 1990b). Further, the factor appears to be specific to the oocyte (Buccione et al., 1990b). Taken together, these results show that stimulation of murine cumulus cell expansion requires a relatively specific factor(s). As bovine oocytes or bovine oocyte conditioned media enable murine cumulus cells to expand, it is likely that both murine and bovine oocytes secrete a similar, if not identical factor(s).

Characterisation of the cumulus expansion enabling factor has been hampered by collection and storage difficulties (Eppig et al., 1993a). In vitro grown oocytes have been used as a potentially large source of cumulus expansion enabling factor (Eppig et al., 1993a). Although these oocytes were capable of stimulating cumulus cell expansion, the amount of cumulus expansion enabling factor produced by these oocytes was about half that of in vivo grown oocytes (Eppig et al., 1993a). In addition to the preliminary characterisation of the murine factor by Eppig et al., (1993a), we have now shown that from the abundant source of preovulatory oocytes from abattoir derived ovaries, the activity of the factor is still present after freezing and storage at −20°C for periods up to at least 1 month. This will allow quantities of media containing the factor to be stored for further purification and elucidation of its structure and function.

Here, a factor has been shown to be secreted by bovine oocytes that enables cumulus cell expansion by murine oocytes. Other paracrine factors may be involved in cumulus cell expansion. A more detailed understanding of these factors will be necessary for the improvement of current in vitro maturation and fertilization systems.

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